UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

CLINICAL RELEVANCE OF SERUM CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4 (CTLA-4) AND ITS CORRELATION WITH THE PRO-INFLAMMATORY CYTOKINES INTERLEUKIN 6 (IL-6) AND TUMOR NECROSIS FACTOR ALPHA (TNF-α) IN FELINE MAMMARY CARCINOMA

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RELEVÂNCIA CLÍNICA DA PROTEÍNA 4 ASSOCIADA AO LINFÓCITO T CITOTÓXICO 4 (CTLA-4) E SUA CORRELAÇÃO COM AS CITOQUINAS PRÓ-INFLAMATÓRIAS INTERLEUCINA 6 (IL-6) E FACTOR DE NECROSE TUMORAL ALFA (TNF-α) NO CARCINOMA MAMÁRIO FELINO

Resumo

A associação entre a expressão da CTLA-4 e o prognóstico no cancro tem sido amplamente investigada, valorizando o papel da inflamação e do microambiente tumoral (TME), do qual mediadores inflamatórios como as citoquinas são uma importante componente não celular. Até à data, não existem estudos sobre reguladores de *checkpoint* imunológico em gatos com carcinoma mamário, nem foram avaliados perfis de citoquinas. Assim, foram investigados pela primeira vez, os perfis séricos da CTLA-4 e das citoquinas pró-inflamatórias IL-6 e TNF-α em 57 gatas com carcinoma mamário e verificada a existência de associações entre os níveis séricos da CTLA-4 e das referidas citoquinas. Os resultados obtidos demonstram que os níveis de CTLA-4 estão aumentados no soro das gatas com carcinoma mamário, quando comparadas com animais saudáveis (P=0.022). Foi também demonstrada uma correlação forte com os níveis séricos do TNF-α (R=0.88, P<0.001) e da IL-6 (R=0.72, P<0.001), reforçando o papel imunomodulatório deste regulador. Adicionalmente foi encontrada uma associação significativa entre os níveis séricos elevados da CTLA-4, e várias características clinicopatológicas menos agressivas: tumores mais pequenos (P<0.001), estadiamento precoce, (P=0.002), ausência de necrose tumoral (P<0.001), sem envolvimento dos linfonodos (P=0.007), sem invasão linfática (P=0.006), com positividade para os receptores hormonais (P=0.007), subtipo não-TN (P=0.041), subtipo não-basal (P<0.001), e baixo índice Ki67 (P=0.001). Os resultados obtidos ainda revelaram uma associação com subtipos específicos de cancro da mama, nomeadamente o HER-2 positivo com sobre-expressão da CTLA-4 (P<0.001) e do TNF-α (P=0.004) e o luminal A com sobre-expressão da IL-6 (P=0.020). Não foi possível confirmar a associação entre os níveis séricos da CTLA-4 e das citoquinas e o tempo de sobrevivência, devido ao tamanho reduzido da amostra. No entanto, os resultados obtidos sugerem um efeito protetor dependente da concentração da CTLA-4 e IL-6 séricos, como evidenciado pelos tempos medianos de sobrevivência mais altos nos grupos CTLA-4^{high} (28 vs 22 meses para o grupo CTLA-4^{low}) e IL-6^{high} (28 vs 19 meses para o grupo IL-6^{low}). Em contraste, o TNF-α parece ser um fator de prognóstico negativo, como sugere o tempo mediano de sobrevivência mais baixo no grupo TNF-$α^{high}$ (16.5 vs 23.5 meses para o grupo TNF-$α^{low}$). Permanece a questão de como o CTLA-4 influencia ou é influenciado pelas citoquinas pró-inflamatórias. A avaliação da expressão tumoral da CTLA-4, dos subtipos de linfócitos T, e dos perfis de macrófagos associados ao tumor e células supressoras da linha mieloide no microambiente tumoral, são aspetos importantes a avaliar em estudos futuros. **Palavras chave**: carcinoma mamário felino, proteína 2 associada ao linfócito t citotóxico, citoquinas pró-inflamatórias, biomarcadores séricos, oncologia comparada

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Abstract

The association between CTLA-4 expression and cancer prognosis has been extensively investigated in recent years, pointing to the link with inflammation, and highlighting the role of the tumor microenvironment (TME), of which inflammatory mediators like cytokines are an important non-cellular component. To the best of our knowledge, no studies on immune checkpoint regulators had been conducted on cats with mammary carcinoma before, nor had cytokine profiles been previously assessed. Thus, we investigated the serum profiles of CTLA-4 and pro-inflammatory cytokines IL-6 and TNF-α in 57 female cats with mammary carcinoma and checked for associations between CTLA-4 and cytokine serum levels. Our results clearly demonstrate that serum CTLA-4 levels are increased in cats with mammary carcinoma when compared to healthy animals (P=0.022). Furthermore, we show a strong positive correlation with TNF-α (R=0.88, P<0.001) and IL-6 levels (R=0.72, P<0.001), advancing the concept of an immunomodulatory role for this regulator in breast cancer pathogenesis. We also show a statistically significant association between higher levels of serum CTLA-4 and less aggressive clinicopathological features: smaller tumors (P<0.001), lower stage (P=0.002), absence of necrosis (P<0.001), no lymph node involvement (P=0.007), no lymphatic vessel invasion (P=0.006), positive hormone receptor status (P=0.007), non-TN status (P=0.041), non-basal status (P<0.001) and low Ki67 index (P=0.001). Our findings further expand this concept by indicating an association with specific breast cancer subtypes, namely, HER-2 positive with CTLA-4 (P<0.001) and TNF-α (P=0.004) and luminal A-like with IL-6 (P=0.020). We could not confirm an association between serum CTLA-4 and cytokines levels and survival due to the small sample size. Nevertheless, our findings suggest a potentially concentration-dependent protective role for serum CTLA-4 and IL-6, as evidenced by higher median survival times in the CTLA-4^{high} (28 vs 22 months for the CTLA-4^{low} group) and IL-6^{high} (28 vs 19 months for the IL-6^{low} group) groups. Conversely, TNF-α seems to be a negative prognostic factor, as shown by the lower median survival in the TNF-$α^{high}$ group (16.5 vs 23.5 months for the TNF-$α^{low}$ group). An intriguing question that remains is how serum CTLA-4 influences or is influenced by the pro-inflammatory cytokines. Assessment of CTLA-4 tumor expression, T-lymphocyte subtypes, and tumor associated macrophages and myeloid derived suppressor cell profiles in the microenvironment, are important features to evaluate in future studies.

Keywords: feline mammary carcinoma, cytotoxic t-lymphocyte associated protein 4, pro-inflammatory cytokines, serum biomarkers, comparative oncology

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List of Abbreviations

AKT – Protein kinase B
APC – Antigen presenting cell
Arg1 – Arginase 1
BRCA – Breast cancer susceptibility gene
CCL22 – C-C motif chemokine 22
CD – Cluster of diferentiation
CK5/6 – Cytokeratin 5/6
COX-2 – Cyclooxygenase 2
cSMAC – Central supramolecular activation cluster
CTLA-4 – Cytotoxic T-lymphocyte associated protein 4
DC – Dendritic cell
DFS – Disease free survival
EGF – Epidermal growth factor
ER – Estrogen receptor
gp130 – Glycoprotein 130
HER-2 – Epidermal growth factor receptor 2
HRP – Horse radish peroxidase
IDO – Indoleamine 2,3-dioxygenase
IFN – Interferon
IL – Interleukin
IL-6Rα – Interleukin 6 receptor alpha subunit
iNOS – Inducible nitric oxide synthase
JAK – Janus kinase
mCTLA-4 – Membrane bound cytotoxic T-lymphocyte associated protein 4
MDSC – Myeloid derived suppressor cell
NFκB – Nuclear factor kappa B
NK – Natural killer cell
NO – Nitric oxide
OS – Overall survival
PDCD4 – Programmed cell death protein 4
PIP3 – Phosphatidylinisitol (3,4,5)-trisphosphate
PR – Progesterone receptor
PTEN – Phosphatase and tensin homolog
RONS – Reactive oxygen and nitrogen species
ROS – Reactive oxygen species

sCTLA-4 – Soluble cytotoxic T-lymphocyte associated protein 4

SOCS3 – Suppressor of cytokine signaling 3

STAT – Signal transducer and activator of transcription

TAM – Tumor associated macrophage

TCR – T-cell receptor

TGF – Transforming growth factor

Th1 – Type 1 T-helper cells

Th17 – Type 17 T-helper cells

Th2 – Type 2 T-helper cells

TIL – Tumor infiltrating lymphocytes

TLR – Toll-like receptor

TMB – 3,3’,5,5’-Tetramethylbenzidine

TME – Tumor microenvironment

TN – Triple negative

TNBC – Triple negative breast cancer

TNF – Tumor necrosis factor

TNFR – Tumor necrosis factor receptor

TP53 – Tumor protein p53

TRADD – Tumor necrosis factor receptor type-1 associated protein

TRAF – Tumor necrosis factor associated factor

Treg – Regulatory T-lymphocyte

VEGF – Vascular endothelial growth factor

1. Introduction

1.1. Feline mammary carcinoma as a model for human breast cancer

Cats have proved to be valuable models for various non-neoplastic (Narfström et al. 2013) and neoplastic diseases (De Maria et al. 2005). Compared with traditional mouse models, they demonstrate more features in common with humans: they share many anatomical and physical similarities, have longer life spans, greater size, a genetically more heterogeneous background, and are exposed to the same environmental risk factors (Cannon 2015). They continue to grow in popularity as pets and experience increasingly higher levels of medical surveillance, making them especially useful models for the study of spontaneous disease and better candidates for use in clinical trials (De Vico and Maiolino 2008).

Several spontaneous feline tumors are currently considered relevant for human cancer studies, including injection-site sarcoma, oral squamous cell carcinoma, lymphoma and mammary carcinoma (De Vico and Maiolino 2008; Cannon 2015; Thomas 2015). Mammary gland tumors are frequently reported as the third most common tumor type affecting female cats and the most common type of cancer in women. Recently they were identified as the most common type in a 10-year retrospective study of feline tumors conducted in Portugal (Garcês et al. 2019). Feline mammary carcinomas are the most representative lesion within this group (Zappulli et al. 2015). Mean age of development is 10-11 years, similar to that described for human breast cancer after adjusting for age, and all breeds may be affected, although evidence points to a hereditary predisposition in the Siamese (Zappulli et al. 2005; Cannon 2015). Similarly, a hereditary predisposition has been observed in women, often associated with mutations at the BRCA1 and BRCA2 genes. Women carrying these mutations are significantly younger at time of diagnosis. Interestingly, the mean age of presentation in Siamese cats is also lower than in other breeds (Zappulli et al. 2005; Cannon 2015).

Feline mammary carcinoma occurs either as single or multiple nodules frequently showing ulceration associated with extensive tumoral necrosis, probably owing to their generally advanced stage at time of diagnosis (Zappulli et al. 2005). They are highly infiltrative and metastasizing, showing a metastatic pattern similar to that described in women (regional lymph nodes, lungs, pleura and liver) (Zappulli et al. 2005).

The influence of steroid hormones on the development of breast cancer is well-known in women and there is evidence of a similar involvement in cats. Intact females have a significantly higher risk of developing disease, as do those exposed to regular and prolonged administration of progestagens (Zappulli et al. 2005). Most cats however tend to have estrogen (ER)-receptor and progesterone (PR)-receptor negative tumors (Cannon 2015). Epidermal

growth factor receptor 2 (HER-2) overexpression is also well documented in human breast cancer often associated with poor prognosis (De Maria et al. 2005). Increased HER-2 expression has been documented in a significant proportion of feline mammary carcinomas, although there is variation among studies as to the degree of overexpression (De Maria et al. 2005; Santos et al. 2013; Cannon 2015). Regardless, taken together with the high level of homology between the feline HER-2 gene transcript and the human sequence this qualifies cats as suitable models (De Maria et al. 2005).

Additional molecular analyses distinguish several other subtypes of breast cancer: luminal A, expressing the luminal epithelial markers (CK7, CK8, CK18, CK19) and with high expression of ER markers and lower expression of proliferation markers; luminal B, expressing the luminal epithelial markers, with lower expression of ER markers and higher expression of proliferation markers; basal-like, negative for hormone receptors (ER, PR, HER-2) and expressing basal markers (CK5, CK6, CK14, CK17, SMA, calponin, vimentin, and p63); HER-2 positive tumors overexpressing the HER-2 receptor; and normal-like, negative to all markers (Goldschmidt et al. 2016). Similar subtypes were identified in cats in a 3-year follow-up study conducted at this institution (Soares et al. 2016b) which identified a higher prevalence of luminal B and triple-negative subtypes, associated with a worse prognosis. Triple-negative breast cancer (TNBC) is also associated with poor prognosis in humans and is especially challenging to treat because of the lack of specific targets. The findings of Soares and colleagues suggest that the cat might also be a suitable model for this highly aggressive subtype.

Studies like the ones conducted by De Maria (2005) and Soares (2016b) reflect a rising interest in the use of immunohistochemical prognostic markers in veterinary oncology. Tissue-based biomarkers, however, often require highly invasive procedures to obtain and may be difficult to include in routine clinical practice. These limitations make biomarkers from liquid biopsies especially valuable. Samples can be collected all through the disease course or before and after specific treatments, to monitor disease progression and predict patient response to therapy (Chakrabarti et al. 2019). Several markers have emerged over the years, some of which have been extensively studied. Serum HER-2 is one such. It can be used to evaluate HER-2 status (i.e. diagnosis), and several studies indicate a role for predicting prognosis and response to treatment (Lüftner et al. 2003). HER-2 serum levels have also been investigated in cats and showed significant association with HER-2 in tissue samples (Soares et al. 2016a) making cats promissing candidates for use in the study of novel serum markers.

The use of cats as a model for human cancers, however, also presents some challenges. Most cat owners are still unwilling to consider enrolling their animals in clinical trials and their use as pre-clinical models for assessment of new drugs is hampered by differences in drug metabolism and pharmacokinetics (Cannon 2015). Cats are known, for

example, to have reduced glucuronidation capacity in comparison to humans, and several chemotherapeutic drugs, including cisplatin, 5-fluorouracil, doxorubicin and ifosfamide have differing toxicities in cats (reviewed in Cannon 2015). Clinical follow-up data is also not always available and some predictors of clinical behavior, namely overall survival (OS) and disease-free survival (DFS; time from surgery to the development of recurrences and/or metastases) rely on information obtained retrospectively through methods that are often imprecise (Zappulli et al. 2015).

When considering the benefits of cats as models in biomedical research it is also important to consider the ethical costs of involving these animals in experiments. The use of novel therapies in the treatment of spontaneous diseases in companion animals might seem more ethically acceptable than in experimentally induced pathologies in animal models. However, the animals enrolled in experimental trials should still be considered as veterinary patients and their management should involve a close partnership between the owners, veterinary practitioners and the veterinarians in the research institutions. To guarantee reliability of results, increase enrolment and, above all, ensure the patient's well-being, an approach based on information, commitment, responsibility and care must be taken (De Vico and Maiolino 2008).

Despite these challenges, feline models remain an exciting prospect in the field of comparative oncology and contribute a diverse range of opportunities to the "One Health" concept, which capitalizes on the integration of biomedical research efforts to achieve better health care for humans and animals. Investigating cancer in cats may additionally generate new insights into aspects of tumor biology that are less accessible in other species (Thomas 2015), such as the role of viruses in malignant transformation and the relationship between inflammation and tumor development.

1.2. Inflammation and tumor development

Inflammation is a well-established risk factor for several cancers. It can contribute to tumor initiation by inducing genetic and epigenetic changes such as point mutations, DNA methylation, and post translational modifications of genes that regulate critical pathways related to cell homeostasis (Hussain and Harris 2007). Injection site sarcoma (ISS), a well-recognized phenomenon in cats particularly associated with vaccine administration, is thought to result from chronic inflammation due to an inappropriate and excessive response to injection or trauma, which causes proliferation and malignant transformation of fibroblasts (Cannon 2015). In Kaposi's sarcoma virus infections, inflammation is also essential for tumor development. Other types of alterations concurring to tumor progression, such as activation of oncogenes and inactivation of tumor suppressors may also trigger the inflammatory cascade,

leading to changes in the cellular microenvironment which favor selection and expansion of cells with growth or survival advantages (called the “intrinsic” pathway, Figure 1). Examples of these adaptive changes include increased expression of antioxidant enzymes, matrix metalloproteinases and growth factor receptors, increased anaerobic respiration and *de novo* synthesis of angiogenic factors (Federico et al. 2007). During tumor promotion, these initiated cells produce inflammatory mediators such as reactive oxygen and nitrogen species (RONS), cytokines, prostaglandins, growth factors and specific microRNAs (Schetter et al. 2009). These, in turn, induce cell proliferation and recruit inflammatory cells, increasing the production of RONS and leading to further DNA damage and reduced DNA repair, perpetuating the cycle (Coussens and Werb 2010).

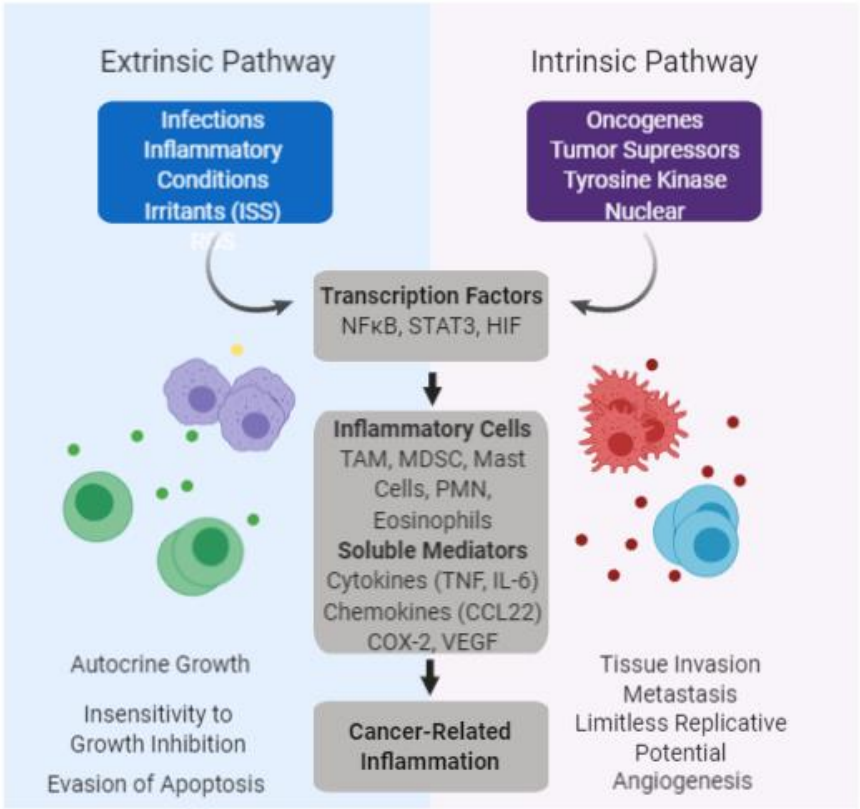

Figure 1. Molecular pathways connecting inflammation and tumor development. ISS – Injection site sarcoma; ROS – Reactive oxygen species; NFκB – Nuclear factor kappa B; STAT – Signal transducers activator of transcription; HIF – Hypoxia inducible factor; TAM – Tumor associated macrophages; MDSC – Myeloid-derived suppressor cells; PMN – Polymorphic nuclear cells; COX-2 – cyclooxygenase 2; VEGF - vascular endothelial growth factor. Redrawn from the original in Denardo, 2017. Created with BioRender.

Key players in cancer related inflammation include transcription factors such as nuclear factor κB (NFκB), signal transducer activator of transcription (STAT)-3, and primary inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α

(Del Prete et al. 2011). NFκB induces the expression of inflammatory cytokines, such as IL-6, adhesion molecules, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS), generating a strong pro-inflammatory microenvironment. It also promotes cell survival and proliferation through the activation of genes regulating cell cycle progression and apoptosis and other pro-tumorigenic changes, including stimulation of angiogenesis by activating vascular endothelial growth factor (VEGF) (Schetter et al. 2009). NFκB activation can follow sensing of microbes or tissue damage by the toll-like receptor (TLR)-MyD88 pathway, inflammatory cytokines TNF-α and IL-1β or can be the result of cell-autonomous genetic alterations in cancer cells (Del Prete et al. 2011). STAT3 is a critical regulator of cytokine, chemokine and growth factor expression. It's persistent activation in tumor cells, either through increased production of positive effectors such as IL-6 or decreased expression of negative regulators such as suppressor of cytokine signaling 3 (SOCS3), in turn activates STAT3 in stromal cells, inducing and maintaining an inflammatory microenvironment (Chang et al. 2015). Activated STAT3 also increases tumor cell proliferation, survival and invasion, while suppressing anti-tumor immunity by promoting pro-tumorigenic pathways like NFκB. Both transcription factors have been shown to play a role in human TNBC, a useful clinical example of the connection between oncogenes and inflammation. In TNBC somatic mutations leading to the inactivation of the tumor suppressor genes tumor protein p53 (TP53) and phosphatase and tensin homolog (PTEN) were implicated in the SOCS3-mediated activation of an IL-6/STAT3/NF κB inflammatory loop (Kim et al. 2014). Mutations in TP53 and PTEN have also been reported in feline mammary carcinoma (Mayr et al. 2000; Ressel et al. 2009; Adega et al. 2016) and considering the high level of sequence homology between the human and feline TP53 and PTEN genes it is reasonable to assume that the mechanisms of tumorigenesis may be similar in the two species. In fact, aberrant activation of the phosphatidylinositol 3,4,5-triphosphate (PIP3)/protein kinase B (AKT)/PTEN pathway, another pathway widely implicated as a driver of tumor development and progression in human breast cancer, was recently shown to be correlated with tumor malignancy, histological differentiation and clinical recurrence in feline mammary carcinoma (Maniscalco et al. 2012).

Despite this overwhelming evidence that inflammation orchestrates a tumor-promoting microenvironment that is intimately linked to tumorigenesis, anti-tumor immunity can also develop to protect the host during tumor development. Data generated in several mouse models which shows that cytokines and immune cells that promote inflammation are potentially bi-functional displaying both tumor-promoting and tumor-suppressive capabilities (reviewed in Chow et al. 2012) supports this notion. Recent developments in immune checkpoint blockade therapy also highlight how important it is to understand the complexity of the immune and

inflammatory systems in the development of cancer and how one's own host responses can help or hinder progression of the disease.

1.3. CTLA-4: a key immune checkpoint regulator

The inflammatory microenvironment surrounding breast cancer cells consists of immune cell infiltrates, cytokines and immune checkpoint molecules that can block anti-tumor immunity (Emens 2012; Yu et al. 2015). Cytotoxic T-lymphocyte associated protein 4 (CTLA-4, CD152), an adhesion molecule from the immunoglobulin superfamily localized on band q33 of human chromosome 2 and on feline chromosome C1, is one of these immune checkpoint molecules. It's expressed exclusively on lymphocytes and shares a pair of ligands expressed on the surface of antigen-presenting cells (APCs), such as dendritic cells (DCs) and B cells, with its homologue, the cluster of differentiation 28 (CD28) receptor. While CD28 interaction with ligands B7-1 (CD80) and B7-2 (CD86), mediates T-lymphocyte co-stimulation in conjunction with T-cell receptor (TCR) signals, CTLA-4 ligand binding, reduces T-lymphocyte activation, forming a negative feedback loop that is essential to the maintenance of immune self-tolerance and homeostasis (Figure 2).

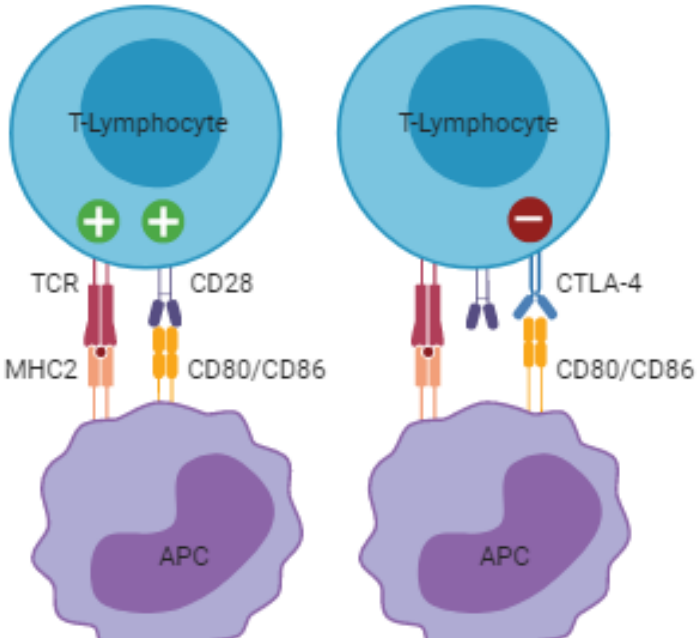

Figure 2. T-lymphocyte activation and inhibition by the immunoglobulin superfamily receptors cluster of differentiation 28 (CD28) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4). After recognition of the MHC:peptide complex by TCR, T-lymphocytes require a second signal for activation which is provided by binding of CD28 to its ligands CD80/CD86 on APCs. This interaction leads to translocation of CTLA-4 to the cell surface. Because CTLA-4 has higher affinity for CD80/CD86 it can interrupt the activation signal delivered by CD28 and deliver its own signal which downregulates T-lymphocyte function. TCR – T cell receptor; MHC – Major histocompatibility complex; APC – Antigen presenting cell. Figure redrawn from the original in Bell et al. 2018. Created with BioRender.

Three isoforms of CTLA-4 have been identified, which result from differential gene splicing: a full-length membrane-bound receptor isoform (mCTLA-4) with an extracellular ligand-binding domain, a transmembrane signal-transducing domain and a cytoplasmic tail; a soluble isoform (sCTLA-4), which does not have the transmembrane domain; and a ligand-independent third isoform, only identified in mice, which lacks the extracellular domain (Wing et al. 2011; Denesha et al. 2014; Yu et al. 2015; Zhang et al. 2016). Feline CTLA-4 shows a high level of sequence homology with the human and murine molecules (86.6% and 76.2% respectively) and the hexapeptide motif (MYPPPY) within the extracellular domain, believed to be responsible for interaction with the B7 ligands, is completely conserved among the studied mammalian species (Ohno et al. 1999) (Figure 3). Upon T-lymphocyte activation, CTLA-4 is transiently translocated to the cell surface where it binds the B7 ligands and initiates inhibitory signals via its intracellular domain (Sharpe and Freeman 2002; Yu et al. 2015). Because CTLA-4 interacts with both B7 ligands, with higher affinity, it can outcompete CD28, attenuating the effector T-lymphocyte response through the inhibition of IL-2 production, blockade of cell cycle progression and therefore, T-lymphocyte proliferation (Walker and Sansom 2015). CTLA-4-expressing cells can also capture B7 ligands from opposing cells by trans-endocytosis and degrade them, resulting in impaired co-stimulation via CD28 (Qureshi et al. 2011).

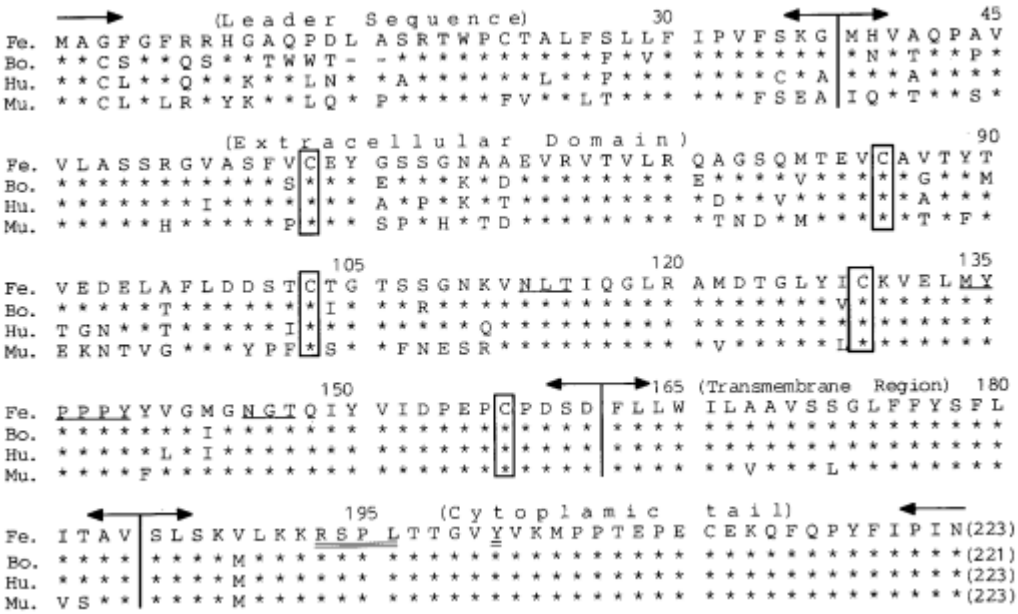

Figure 3. Alignment of feline (Fe) CTLA-4 amino acid sequence with homologues from bovine (Bo), human (Hu) and mouse (Mu) species. The hexapeptide MYPPPY ligand binding motif on the extracellular domain is underlined. Original in Ohno et al. 1999.

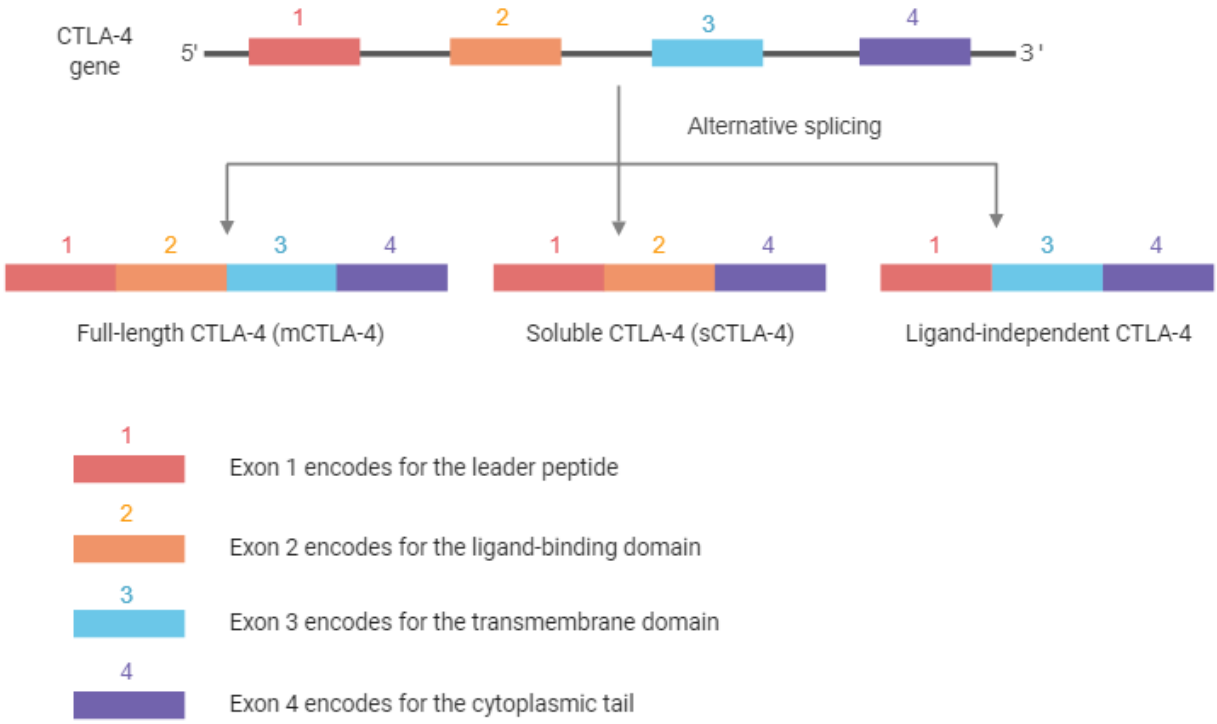

Figure 4. Generation of full-length, soluble and ligand-independent cytotoxic T-lymphocyte associated protein 4 (CTLA-4) mRNA. The CTLA-4 gene encodes a transcript with four exons. Splicing generates the full-length transcript (mCTLA-4). Alternative splicing generates two shorter transcripts: the sCTLA-4 transcript that skips exon 2 (ligand-binding domain) and a ligand-independent transcript, only identified in mice, that skips exon 3 (transmembrane domain). Figure redrawn from the original in (Simone and Saverino 2009). Created with BioRender.

sCTLA-4 is generated by alternative splicing of the CTLA-4 mRNA in which the exon that encodes the transmembrane region (exon 2) is spliced out (Figure 4). The deletion causes a shift in the reading frame, producing a unique cytoplasmic tail that is unique to the sCTLA-4 molecule. The alternative splicing also results in the loss of the membrane proximal cysteine residue required for covalent homodimerization, making sCTLA-4 a monomer (Oaks et al. 2000). Both the full-length and the sCTLA-4 transcripts are expressed in CD4+ T-lymphocytes but mCTLA-4 is the predominant among CD8+ subsets of T-lymphocytes, as well as B-lymphocytes. mCTLA-4 is also the predominant transcript on activated T-lymphocytes, however on resting cells or at the post-activated state sCTLA-4 predominates (Oaks et al. 2000).

sCTLA-4 is secreted in a similar manner to mCTLA-4. Upon TCR stimulation, secretory granules are translocated to the central supramolecular activation cluster (cSMAC) within the immunological synapse releasing sCTLA-4 which can interact with the B7 ligands, excluding CD28 from the cSMAC thus inhibiting early T-lymphocyte responses to antigens (Wing et al. 2011; Yu et al. 2015). Translocation is fully dependent on ligand binding but does not require high amounts of ligand in the cSMAC which indicates that sCTLA-4 can control T-lymphocyte

activation even when access to co-stimulatory molecules is limited (Wing et al. 2011). sCTLA-4 signaling may also affect the adhesion and motility of T-lymphocytes to APCs, inhibiting the TCR-mediated signal through dephosphorylation of the TCR signaling proteins via it's cytoplasmic tail, and may induce production of indoleamine 2,3-dioxygenase (IDO) and immunosuppressive kynurenine in the latter (Ward et al. 2013; Pico de Coaña et al. 2014). IDO is an enzyme system that depletes the amino acid L-tryptophan, establishing a microenvironment which impairs the growth and survival of T-lymphocytes. Finally, sCTLA-4 may induce nuclear localization of the transcription factor Forkhead box (Fox)O3, which inhibits production of inflammatory cytokines, including IL-6 (Dejean et al. 2009; Ward et al. 2013), and can induce increased production of IL-10, an immunosuppressive cytokine (Dahal et al. 2016), thereby constraining T-lymphocyte survival. Recently sCTLA-4 was also implicated in the induction of the translational inhibitor programmed cell death-4 (PDCD4) as a result of FoxO1 nuclear re-localization, which attenuates effector T-lymphocyte responses (Lingel et al. 2017).

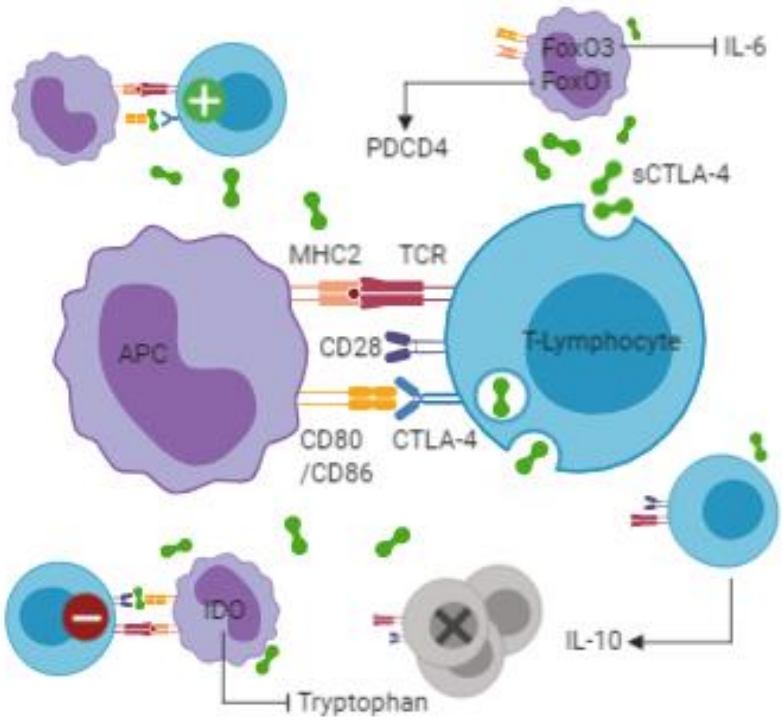

Figure 5. Possible mechanisms of immune regulation by soluble cytotoxic T-lymphocyte associated protein 4 (sCTLA-4). Interference with CTLA-4-lingand interactions enhances T-lymphocyte reactivity. By contrast interference with CD28-ligand interactions may result in T-lymphocyte inhibition. sCTLA-4 may also induce nuclear localization of FoxO3 and FoxO1 with consequent inhibition of inflammatory cytokine production and induction of PDCD4. Finally, sCTLA-4 may induce expression of IDO and immunosuppressive cytokine IL-10 establishing a microenvironment which impairs the growth and survival of T-lymphocytes. APC – Antigen presenting cell; MHC – Major histocompatibility complex; TCR – T cell receptor; CD – Cluster of differentiation; IL – Interleukin; IDO – Indoleamine 2,3-dioxygenase; PDCD – Programmed cell death; Fox – Forkhead box**.** Figure redrawn from the original in Dahal et al. 2018. Created with BioRender.

Elevated serum CTLA-4 levels have been reported in several cancers, including esophageal (Zhang et al. 2016), lung (Liu, Xie, et al. 2017), and breast carcinoma (Erfani et al. 2010). Furthermore, a molecular study of CTLA-4 genotypes and haplotypes by Erfani and colleagues, among others, clearly demonstrated association of CTLA-4 gene variants with cancer. Indeed, CTLA-4 expression appears to be an important mechanism of tumor immune evasion. Deletion of the CTLA-4 gene in Treg cells in mice was shown to produce potent tumor immunity (Wing et al. 2008). Up-regulated expression of CTLA-4 in tumor cells was also recently identified as one of the three most prevalent mechanisms of immune evasion in human breast cancer, the other two being the presence of immunosuppressive factors (i.e. IL-10, transforming growth factor beta – TGF-β, C-C motif chemokine 22 – CCL22), and tumor expression of a soluble decoy receptor (DcR3) which binds to FasL and inhibits FasL-induced apoptosis (Bou-Dargham et al. 2018). Taken together, these findings show that CTLA-4 plays a crucial role in suppression of tumor immunity.

However, the clinical implications of CTLA-4 in the tumor microenvironment are still controversial. Various studies indicate increased levels of sCTLA-4 in several autoimmune diseases (Simone et al. 2014). A recent study on breast cancer patients found an association between elevated sCTLA-4 levels and improved survival (Liu, Hu, et al. 2017) and several other studies have showed significant correlations between CTLA-4 and OS in non-small cell lung carcinoma, nasopharyngeal carcinoma, esophageal carcinoma, malignant hematologic diseases, glioblastoma and malignant pleural mesothelioma (Liu, Xie, et al. 2017). These findings seem counterintuitive because an increase in sCTLA4 should inhibit T-lymphocyte activity. Some researchers have suggested as an explanation that in the resting T-lymphocytes in which only sCTLA-4 is expressed, CD28-ligand interactions are inhibited. But in a later phase, where mCTLA4 is overexpressed, sCTLA4 interferes with mCTLA-4 ligand interactions, enhancing T-lymphocyte reactivity by preventing the transduction of inhibitory signals (Saverino et al. 2007; Pérez-García et al. 2013; Simone et al. 2014). It has also been suggested that CTLA-4 can mediate negative signal into tumor cells, comparable to those observed in T-lymphocytes (Salvi et al. 2012). Salvi and colleagues observed that established non-small cell lung carcinoma cell lines undergo apoptotic death upon CTLA-4 engagement with soluble B7 (CD80/CD86) ligands. They hypothesize that CTLA-4 expressed by tumor cells may interact with B7 ligands expressed by cells of the tumour micro-environment, thus leading to inhibition of lung cancer cell proliferation and/or induction of apoptotic cell death. These findings may support a role for CTLA-4 as a negative regulator of tumor proliferation, important for cancer biology.

1.4. Cellular components of the Tumor microenvironment: the role of Tregs, TAMs and MDSCs

Most solid tumors contain several subtypes of immune cell infiltrates, including both myeloid- and lymphoid-lineage cells. Human breast cancer, in particular, shows significant levels of tumor infiltrating lymphocytes (TILs). Similarly, lymphocytic infiltration is a common finding in feline mammary carcinoma, though its functional role is not yet fully established (Wiese, Thaiwong, Yuzbasiyan-Gurkan, & Kiupel, 2013). TIL populations dominated by T-lymphocytes (CD3+) are the most commonly reported (Ruffell et al., 2011), being usually associated in some molecular subtypes, namely TNBC and HER-2 positive, with improved survival (Adams et al., 2014; Desmedt et al., 2014; Dieci et al., 2015; Loi et al., 2013; Stanton & Disis, 2016). However, the phenotype of the T-lymphocyte response can influence clinical outcome. While type 1 CD4+ T-helper (Th1) lymphocytes and CD8+ cytotoxic T-lymphocytes are generally associated with a favorable prognosis, type 2 CD4+ T-helper (Th2) lymphocytes inhibit effector T-lymphocyte responses and support proliferation of B-lymphocytes, promoting an anti-inflammatory immune response that may enhance tumor growth (Stanton & Disis, 2016; Ward et al., 2013; Zitvogel, Galluzzi, Kepp, Smyth, & Kroemer, 2015).

Th2 regulatory T-lymphocytes (Tregs) are a subset of CD4+ T-lymphocytes that highly express the IL-2 receptor α chain (CD25) and FoxP3. Tregs also express CTLA-4 whose expression is controlled by FoxP3 and which in Tregs, unlike other T-lymphocyte subsets, is expressed constitutively (reviewed in Wing et al. 2011; Ward et al. 2013). Although their main function is to prevent autoimmune disorders by suppressing effector T-lymphocyte activation, Tregs are known to highly infiltrate various tumor types in both humans and felines (Sparger et al. 2018). Several studies show that Tregs can suppress tumor specific T-lymphocyte immunity, contributing to tumor growth, invasion and metastasis and reduced survival (Curiel et al. 2004; Tan et al. 2011; Emens 2012).

Tregs suppress effector T-lymphocytes via several mechanisms, including Fas/Fas ligand (FasL)-mediated apoptosis, granzyme B/perforin-mediated cytotoxicity and IL-2 deprivation through expression of high levels of CD25 (Pandiyan et al. 2007; Wang et al. 2017). Another highly relevant mechanism whereby Tregs are thought to control effector T-lymphocytes is the CTLA-4-dependent downregulation of B7 ligands on DCs upon antigenic stimulation which is significantly impaired in mice with Treg-specific deficiency of CTLA-4 (reviewed in Wing et al. 2011). CTLA-4 interaction with DCs can also induce expression of IDO (Adams et al. 2014), providing further evidence that CTLA-4 is vital for Treg-lymphocyte mediated suppression. Tregs also produce cytokine IL-35, VEGF and TGF-β which act together to promote angiogenesis and prevent the activation of adaptive and innate immune

cells and may induce polarization of M2 macrophages (Jarnicki et al. 2006; Collison et al. 2007; reviewed in Wang et al. 2017).

It should be noted, however, that Treg infiltration can correlate with a positive prognosis in certain types of cancer. A study in a mouse model of colorectal cancer, showed that under the influence of IL-10, Tregs prevented the development of tumors and rapidly induced tumor regression, at least in part through the inhibition of COX-2 (Erdman et al. 2005). Studies conducted on head and neck, esophageal and hematologic cancers came to similar conclusions (Shang et al. 2015). Tregs have also been shown to suppress inflammation triggered by innate immune cells, such as macrophages and monocytes, in mice and in human cancers (Shang et al. 2015) and seem to be the primary source of sCTLA-4 (Ward et al. 2013) which correlates with improved prognosis in breast cancer patients. These findings raise the possibly of a protective role for Tregs in cancer and warrant further investigation.

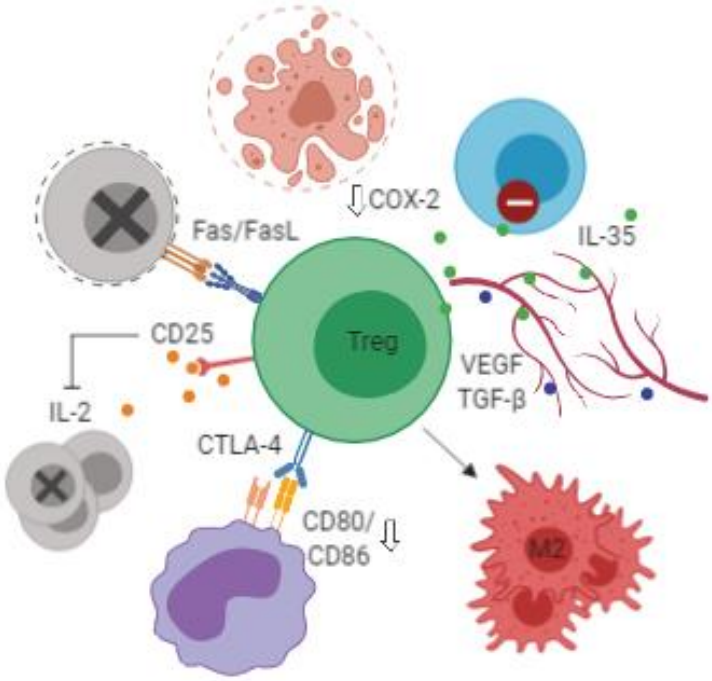

Figure 6. Functions of regulatory T-lymphocytes (Tregs) in the tumor microenvironment. Tregs are capable of suppressing effector T-lymphocyte responses through several mechanisms, including Fas/FasL-mediated apoptosis, granzyme B/perforin-mediated cytotoxicity, IL-2 deprivation through expression of high levels of CD25 and expression of immunosuppressive cytokines like IL-35. Tregs also reduce T-lymphocyte co-stimulatory signals through depletion of CD80/CD86 on dendritic cells via CTLA-4 mediated trans-endocytosis. Tregs may induce polarization of M2 macrophages and promote angiogenesis by secretion of VEGF and TGF-β. Tregs may also have tumor-suppressive effects, for example via inhibition of COX-2. IL – Interleukin; CD – Cluster of differentiation; FasL – Fas ligand; COX – Cyclooxygenase; VEGF – Vascular endothelial growth factor; TGF – Transforming growth factor; CTLA – cytotoxic T-lymphocyte associated protein. Figure redrawn from the original in Wang et al. 2017. Created with BioRender.

Similar to Tregs, the role of IL-17 producing CD4+ cells (Th17) in the pathogenesis of cancer needs further elucidation. A study on melanoma in a mouse model found that IL-17 signaling was critical for tumor development, with direct effects on tumor and stromal cell-induced production of IL-6 which led to activation of STAT3 (Wang et al. 2009). Dysregulation of the IL-6-mediated STAT3 signaling pathway is also closely related to the development of breast cancer in humans. Other studies, however, have revealed a potential tumor-suppressive role for this cell type. Th17 cells promoted tumor-specific cytotoxic T-lymphocyte activation in a model of lung melanoma (Martin-Orozco et al. 2009) and were positively associated with a more favorable prognosis in human breast carcinoma (Yang et al. 2012).

Myeloid-lineage cells like tumor associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) also play a role in tumor development. In contrast to T-lymphocytes, TAM infiltration is often associated with poor prognosis in human breast cancer (DeNardo et al. 2009; reviewed in Mantovani et al. 2017) and canine mammary tumors (Raposo et al. 2014). These macrophages usually exhibit an M2 phenotype induced by Th2 lymphocytes (Jackute et al. 2018) and may block T-lymphocyte responses through the production of immunosuppressive molecules IL-10, TGF-β and the arginine-degrading enzyme arginase-1 (Arg-1) as well as induce differentiation and recruitment of Tregs via C-C motif chemokine 22 (Curiel et al. 2004; Brown et al. 2017). Moreover, they may support tissue repair and angiogenesis through the production of VEGF or epidermal growth factor (EGF) (Brown et al. 2017). MDSCs, a heterogeneous group of immature cells also seem to be significantly increased in the peripheral blood of breast cancer patients, associated with more aggressive molecular subtypes such as TNBC, advanced stage and positive lymph node status (Safarzadeh et al. 2019). They can influence the tumor microenvironment through multiple mechanisms, including production of reactive oxygen species (ROS) which induces the loss of the TCR ζ-chain leading to T-lymphocyte anergy, and production of Arg1, and IDO, all of which lead to cell cycle arrest of T-lymphocytes (Kumar, Patel, Tcyganov, & Gabrilovich, 2016; Markowitz, Wesolowski, Papenfuss, Brooks, & Carson, 2013; Pico de Coaña, Masucci, Hansson, & Kiessling, 2014). MDSCs can also induce differentiation of CD4+ T-lymphocytes into Tregs and Treg expansion through the secretion of inhibitory cytokines IL-10 and TGF-β. (Markowitz et al. 2013; Pico de Coaña et al. 2014; Kumar et al. 2016).

MDSCs and TAMs, however, do not suppress all aspects of antitumor immune responses. Like TAMs, MDSCs exhibit two distinct phenotypes, the tumor-suppressing M1-like and the tumor-promoting M2-like (Ma et al. 2011). Whereas M2 MDSCs inactivate effector T-lymphocytes and recruit Tregs, M1 MDSCs have the opposing effect. They express higher quantities of pro-inflammatory cytokines and can activate NK cells to produce high amounts of interferon (IFN)-γ (Nausch et al. 2008). IFN-γ induces iNOS expression which generates high amounts of nitric oxide (NO). Because NOs cellular activities are concentration-dependent,

with higher levels producing cytotoxicity and antitumorigenic effects (Hussain and Harris 2007), this suggests that M1 MDSCs may have direct tumor-killing activities. Unlike M2 TAMs, the presence of M1 TAMs in the tumor microenvirnoment is associated with increased survival (Jackute et al. 2018). They also direct T-lymphocytes towards Th1 tumor-suppressive responses and interact with NK cells promoting apoptosis in tumor cells through expression of iNOS or TNF-α (Cui et al. 1994; O'Sullivan et al. 2012). Finally, a study on the effects of myeloid-derived VEGF in a mouse model of mammary tumorigenesis, determined that although this factor does increase vascular density in tumors, this change acts to retard not promote tumor progression, as was previously thought (Stockmann et al. 2008).

It is evident that many immune cell types are capable of displaying both tumor-promoting and tumor-suppressive capabilities. However, the cellular and molecular mechanisms that positively or negatively regulate their phenotype and biological functions are not yet fully understood and further study is warranted.

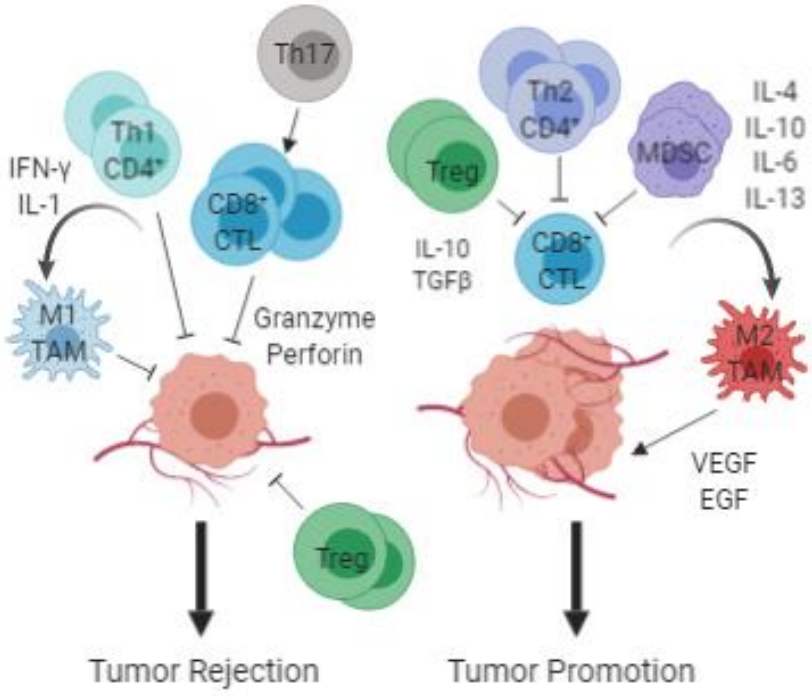

Figure 7. Contrasting functions of immune cells in the tumor microenvironment. Th1 $CD4^+$ and $CD8^+$ T-lymphocytes may directly regulate tumor cell cytotoxicity, while indirectly polarizing immune cells, such as M1 tumor associated macrophages (TAM) and Th17 T-lymphocytes, towards tumor suppression. Th2 CD4+ T-lymphocytes, regulatory T-lymphocytes (Treg) and myeloid derived suppressor cells (MDSC) in contrast, may suppress CD8+ cytotoxicity and induce polarization of immune cells, such as M2 TAMs, which provide a rich proangiogenic and pro-tumoral microenvironment. CTL – Cytotoxic T-Lymphocyte; IFN – interferon; IL – interleukin; TGF – Transforming Growth Factor; VEGF – Vascular Endothelial Growth Factor; EGF – Epidermal Growth Factor. Figure redrawn from the original in (DeNardo and Coussens 2007). Created with BioRender.

1.5. Soluble mediators of cancer related inflammation: TNF-α and IL-6

Cytokines are a non-cellular component of the tumor inflammatory microenvironment that can exert rather paradoxical effects during cancer development. The action of inhibitory cytokines has long been implicated in the lack of an effective immune response observed in breast cancers. They also have been reported to promote tumor growth and aggressiveness by influencing aromatase activity and estrogen synthesis directly in the tumor vicinity (reviewed in Knüpfer and Preiß 2007). Certain cytokines, however, have been demonstrated to promote the generation and/or efficacy of anti-tumor effector cells, including DCs and NK cells, causing inhibition of tumor growth and even tumor regression (Knüpfer and Preiß 2007). Whether they exert tumor-promoting or tumor suppressive effects is highly dependent on a number of factors, including the array of cytokines present, their relative concentration, and presence of other modulating factors, such as cytokine receptor expression patterns and the activation state of the cells that express them (Knüpfer and Preiß 2007).

Briefly, cytokines are small pleiotropic proteins that act by altering the function of their target cells in a paracrine or autocrine manner. They are mainly secreted by lymphocytes and macrophages and can be broadly classified as pro-inflammatory or anti-inflammatory. Of the pro-inflammatory cytokines, TNF-α and IL-6 have emerged as central players linking inflammation and cancer.

TNF-α is a member of the TNF cytokine superfamily and is a key molecule regulating inflammation and host defense. It is predominantly produced by immune cells, namely macrophages, T-lymphocytes and NK cells, but also, in low amounts, by fibroblasts, smooth muscle cells and tumor cells (Tse et al. 2012). There are two TNF receptors, TNF receptor type 1 (TNFR1), which is ubiquitously expressed and TNF receptor type 1 (TNFR2), which is mainly expressed on innate immune cells. Activation of TNF receptors can trigger NF-κB and downstream immunosuppressive survival pathways or can activate caspase 8 and the associated apoptotic signal (Wang and Lin 2008) (Figure 8). These activities vary under different physiological conditions and in a cell-type-dependent manner. For example, in rapidly regenerating tissues, TNF-induced NF-κB activity is anti-tumorigenic, whereas in slowly regenerating tissues it seems to be pro-tumorigenic (Wang and Lin 2008). There is also evidence that while chronic synthesis of low amounts of TNF-α promotes tumor growth and angiogenesis, higher doses may stimulate antitumor immunity, induce necrosis of tumor cells, and trigger vascular collapse (Tse et al. 2012). It is likely that differential expression of the TNF receptors is also involved. TNFR1 is a death domain-containing receptor and transduces both proapoptotic and prosurvival signals. TNFR2 does not possess a death domain and is mainly responsible for the promotion of proliferation, although it can mediate a cell death signal which may be indirect through TNFR1 (Wang and Lin 2008). In breast cancer, recent investigations

strongly suggest that chronic TNF-α expression supports tumor growth. Expression of TNF-α in inflammatory breast carcinoma correlated with increased tumor grade and lymph node involvement, and patients with more progressed tumor phenotypes were shown to have significantly higher serum concentrations of TNF-α (Ben-Baruch 2003). However, it cannot be ascertained from these studies whether the elevated TNF-α contributes to disease progression, or is a reflection of advanced disease, and more research into its clinical diagnostic and prognostic utility is required.

IL-6 is produced mainly by myeloid cells, including monocytes and macrophages, but also T-lymphocytes, fibroblasts and tumor cells (Fisher et al. 2015). As a secreted protein, IL-6 can be detected in serum, and increased levels have been found in breast cancer patients, associated with worse prognosis (Knüpfer and Preiß 2007). IL-6 signaling is initiated through binding to the IL-6 receptor, a heterodimer consisting of the IL-6 receptor α subunit (IL-6Rα; CD126) and a glycoprotein 130 (gp130) β subunit, whose activation triggers phosphorylation of the Janus kinases (JAK) and its downstream effectors, including the STAT proteins STAT1 and STAT3 (Figure 8). STAT phosphorylation allows dimerization, nuclear translocation, and activation of specific target genes which are mainly involved in cell cycle progression and suppression of apoptosis (Lin and Karin 2007). STAT3 has a predominant role in IL-6 signal transduction and its roles in tumor cell proliferation and survival are well documented (Kim et al. 2014; Chang et al. 2015). Chang and colleagues, for example, report that STAT3 activation initiated by IL-6 promotes recruitment of myeloid cells and induces their ability to express growth factors in a feed-forward loop that positively regulates angiogenesis and induces metastasis. While the predominant view of IL-6 in breast cancer is as a driver of malignancy, recent studies have highlighted its beneficial role in promoting anti-tumor immunity. IL-6 plays a vital role in the development of T-lymphocyte responses, being required for T-lymphocyte priming, the induction of a productive IFN-γ response, protection of T-lymphocytes from the suppressive activities of Tregs, and the acquisition of the ability to provide help to B cells (Fisher et al. 2015). IL-6 signaling also influences lymphocyte trafficking to lymph nodes and to tumor tissues stimulating anti-tumor activities within the tumor microenvironment (Fisher et al. 2015). Taken together, these findings underscore the pleiotropic characteristic of this cytokine and further studies are necessary to elucidate its role in tumor development and progression.

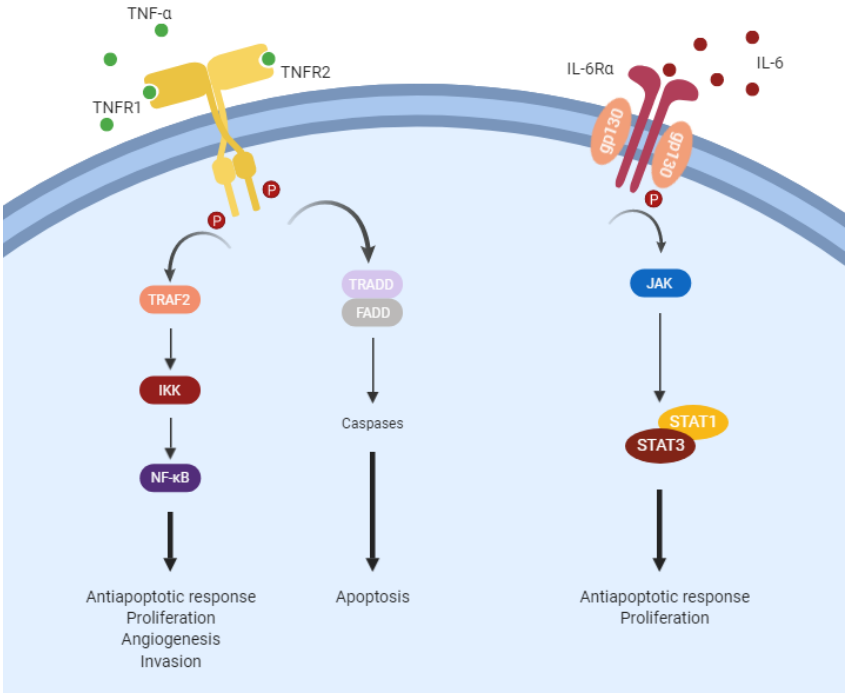

Figure 8. Signal transduction pathways and major biological responses of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). TNFR – Tumor Necrosis Factor Receptor 1; gp – glycoprotein; TRAF – TNF receptor associated factor; TRADD – TNF receptor type 1-associated death domain; JAK – Janus Kinase; STAT – Signal Transducer and Activator of Transcription. Redrawn from the original in Lin and Karin 2007. Created with BioRender.

2. Retrospective Study

2.1. Objective

Several reports have indicated that CTLA-4 is elevated in the sera from patients with several inflammatory human disorders. The association between CTLA-4 and cancer has also been extensively investigated in recent years. These investigations often point to the link between cancer and inflammation, and the role of the tumor microenvironment in cancer initiation, promotion and progression. Immune cells and inflammatory mediators, such as pro-inflammatory cytokines, are an important part of this microenvironment. In the present study, our main objective was to investigate the profiles of serum CTLA-4 and pro-inflammatory cytokines IL-6 and TNF-α in cats with mammary carcinoma. In addition, we sought to determine whether an association between CTLA-4 and pro-inflammatory cytokine serum levels exists. We hope our findings will contribute to the clarification of the complex interactions that occur within the mammary tumor microenvironment and further validate the cat as a model for the study of human breast cancer.

2.2. Materials and Methods

2.2.1. Study Population

Sera from 57 female cats were used in this study. All animals had a fully documented history of feline mammary carcinoma and were followed up at the Faculty of Veterinary Medicine Teaching Hospital (HEV) between June 2011, and September 2013. Available historical data included age, clinical stage (TMN), malignancy grade, tumor burden and size, regional lymph node involvement, presence of tumor necrosis, lymphatic vessel invasion, lymphocyte infiltration or cutaneous ulceration and histopathological classification (ER status, PR status, HER-2 status, basal status, Ki67 index) (Table 1). Serum samples were collected at time of admission, aliquoted and stored at -80ºC. Serum samples from twelve healthy cats were used as controls for the cytokine and sCTLA-4 analysis.

Table 1. Clinicopathological characteristics of female cats with mammary carcinoma (*n*=57). LN – Lymph Node; LVI – Lymphatic Vessel Invasion; LI – Lymphocyte Infiltration; ER – Estrogen Receptor; PR – Progesterone Receptor; HER – Epidermal Growth Factor Receptor; TN – Triple Negative; ND – Not Determined.

Clinical feature	Number (%)	Clinical feature	Number (%)	Clinical feature	Number (%)	Clinical feature	Number (%)
Age (years)		**Size (cm)**		**Necrosis**		**HER-2**	
<8	4 (7)	<2	20 (35)	No necrosis	15 (26)	**status**	
8-12	31 (54)	2-3	20 (35)	Necrosis	42 (74)	Negative	45 (79)
>12	22 (39)	>3	17 (30)	**Ulceration**		Positive	12 (21)
Stage		**LN**		No ulceration	50 (88)	**TN status**	
I	15 (26)	**status**		Ulceration	7 (12)	Non-TN	42 (74)
II	7 (12)	Negative	35 (62)	**Ki67 index**		TN	15 (26)
III	31 (54)	Positive	18 (32)	Low (<14%)	18 (32)	**Basal status**	
IV	4 (7)	ND	4 (7)	High (>14%)	38 (66)	Non-basal	48 (84)
Grade		**LVI**		ND	1 (2)	Basal like	8 (14)
1	3 (5)	No LVI	50 (88)	**ER status**		ND	1 (2)
2	8 (14)	LVI	7 (12)	Negative	39 (68)		
3	46 (81)	**LI**		Positive	18 (32)		
Burden		No LI	16 (28)	**PR status**			
Single tumor	21 (37)	LI	39 (68)	Negative	30 (53)		
Multiple tumors	36 (63)	ND	2 (4)	Positive	27 (47)		

2.2.2. Measurement of serum CTLA-4 and cytokine levels

Serum samples were kept frozen at -80ºC and thawed shortly before determination of CTLA-4, TNF-α and IL-6. Commercially available immunoassay kits from R&D Systems (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturers' instructions. CTLA-4 levels were determined with the Mouse CTLA-4 DuoSet® ELISA immunoassay kit (code DY476); TNF-α levels were determined with the Feline TNF-α DuoSet® ELISA

immunoassay kit (code DY2586); and IL-6 levels were determined with the Feline IL-6 DuoSet® ELISA immunoassay kit (code DY2305). Given the high level of sequence homology between the feline and murine CTLA-4 molecules (76.2%) we estimated that the murine kit would be adequately sensitive. All kit components were stored at 4ºC. A seven-point standard curve was prepared for each assay by making serial dilutions from a stock of recombinant mouse CTLA-4, feline TNF-α and feline IL-6 provided in the kits. The immunoassays use a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) technique (Figure 9). Briefly:

- A 96-well microplate is prepared by adding Capture Antibody to each well, after which the plate is sealed and incubated at room temperature overnight. The antibody is removed, and unbound molecules washed away by washing the plate three times with wash buffer;
- To prevent nonspecific binding the plate is blocked by adding 1% BSA in PBS to each well, and the plate is sealed and incubated at room temperature for one hour. After incubation, blocking agent is removed and the plate washed three times as before with wash buffer.
- Diluted serum samples are added to each well, and the plate is sealed and incubated at room temperature for two hours. After incubation, samples are removed, and unbound molecules washed away by washing three times;
- Biotinylated Detection Antibody is added to each well, and the plate is sealed and incubated at room temperature for another two hours. After incubation, antibody is removed, and unbound molecules washed away by washing three times;
- A working solution of Streptavidin Conjugated to Horseradish Peroxidase (HRP) is added to each well, and the plate is sealed and incubated for 20 minutes at room temperature. After incubation, Streptavidin-HRP is removed, and the plate washed three times as before with wash buffer. During the last wash, the substrate solution is prepared by mixing equal volumes of 3,3',5,5'-tetramethylbenzidine and H_2O_2 solutions.
- Substrate solution is added to each well and incubated for 20 minutes at room temperature.
- Following color development, sulfuric acid is added to stop the reaction.

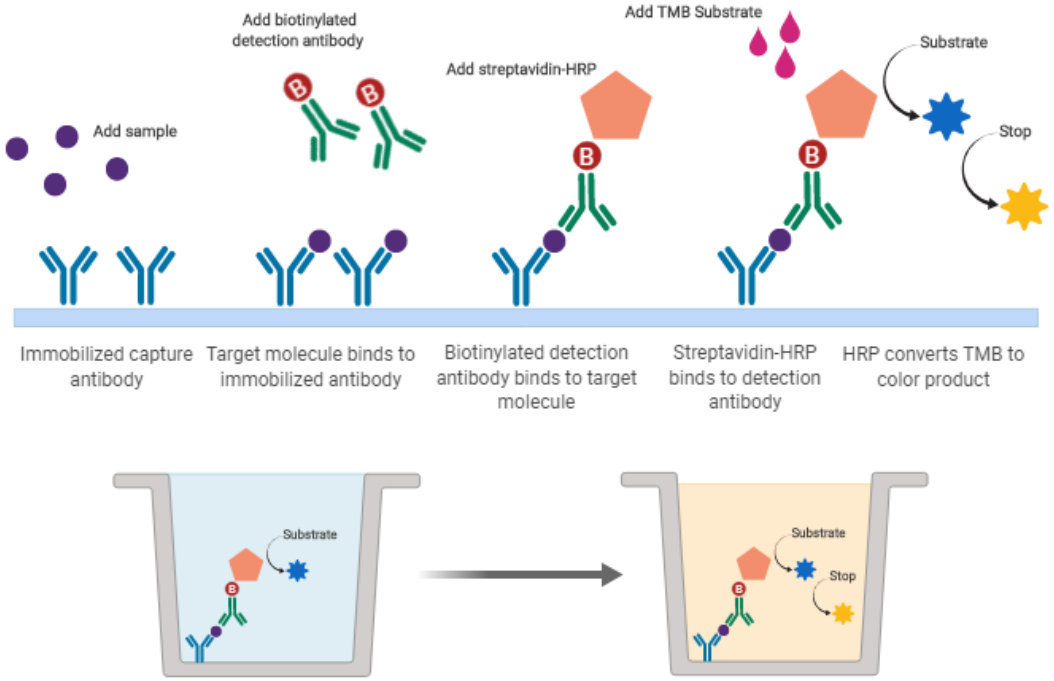

Figure 9. Sandwich enzyme-linked immunosorbent assay (ELISA) technique. HRP – Horse radish peroxidase; TMB – 3,3',5,5'-Tetramethylbenzidine. Created with BioRender.

The optical density was determined using a FLUOstar OPTIMA microplate reader from BMG Labtech, set to 450 nm. To correct for optical imperfections in the plate a second reading was performed at 570 nm and readings were subtracted from the readings at 450 nm. The data were linearized by plotting the log of the mean absorbance against the log of the concentration using Microsoft® Excel® version 1904 for Windows (Microsoft Corporation, Redmond, WA, USA). CTLA-4, TNF-α and IL-6concentrations were determined using the curve fit equation ($y = mx + c$) generated. The correlation coefficient between the fitted data and the actual data was greater than 0.99 for all assays.

2.2.3. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 8.11 for Windows (GraphPad Software, La Jolla, CA, USA). The values $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant. A Kruskal-Wallis test was used to assess significance between serum cytokine and serum CTLA-4 levels and clinicopathological data. Pearson correlation was used to assess the correlation between CTLA-4 and IL-6/TNF-α serum levels. Survival curves were plotted using the Kaplan–Meier (KM) method and the statistical significance between groups determined by the Log-rank test. Receiver operating characteristic (ROC) analysis was used to determine optimal cut-point values for the cytokines.

2.3. Results

2.3.1. Serum CTLA-4 levels

CTLA-4 levels were detectable in 23 (43%) of the 54 cats assessed, showing a median of 459.4 pg/mL when detectable (range 77–999.3 pg/mL). In the following analysis, CTLA-4 serum level is considered to be 0 pg/mL for the 23 patients whose serum level was below the detection limit (31.3 pg/mL). The data were tested for associations with clinicopathological criteria (Table 1). Serum CTLA-4 levels in the cats with mammary carcinoma were significantly higher than those in the healthy group (*P*=0.022; Figure 10)

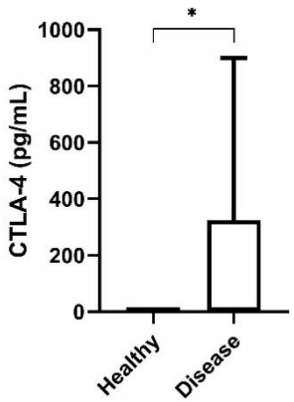

Figure 10. Box plot analysis of serum cytotoxic T-lymphocyte associated protein 4 (CTLA-4) levels in healthy cats and cats with mammary carcinoma. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

Serum CTLA-4 levels were significantly increased in older cats (*P*=0.009; Figure 11a), cats with stage I (*P*=0.002; Figure 11b) and stage II (*P*=0.049; Figure 11b) tumors, smaller tumors (*P*<0.001; Figure 11c), no tumor necrosis (*P*<0.001; Figure 11d), no lymphatic vessel invasion (*P*=0.006; Figure 11e) and no lymph node involvement (*P*=0.007; Figure 11f).

No significant correlation was found between CTLA-4 serum levels and either tumor grade (*P*=0.061), tumor burden (*P*=0.523), lymphocyte infiltration (*P*=0.141) or cutaneous ulceration (*P*=0.056) (data not shown).

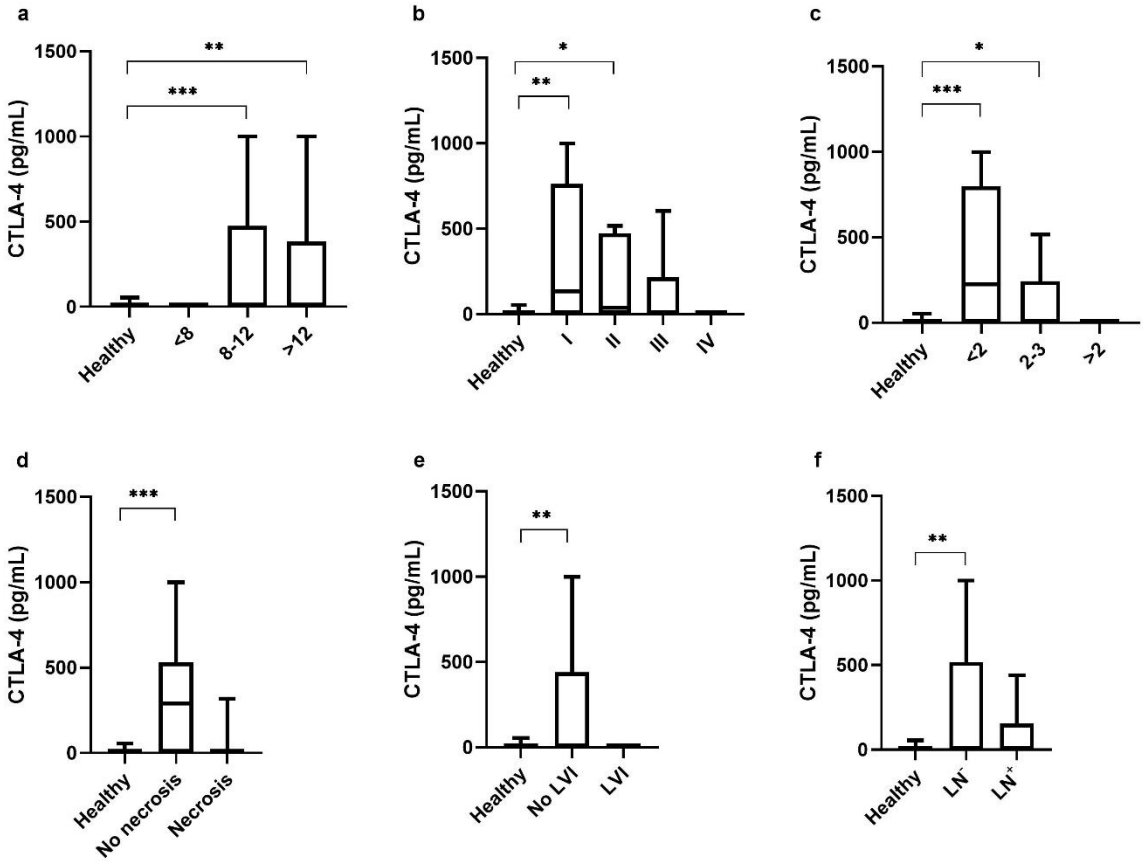

Figure 11. Box plot analysis of serum cytotoxic T-lymphocyte associated protein 4 (CTLA-4) levels and their association with clinicopathological parameters: a) age (years); b) clinical stage; c) tumor size (cm); d) tumor necrosis; e) lymphatic vessel invasion (LVI); f) lymph node status (LN). * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

Serum CTLA-4 levels were also significantly increased in cats with ER-negative (P=0.009; Figure 12a), PR-positive (P=0.007; Figure 12b), HER-2-positive (P<0.001; Figure 12c), non-TN (P=0.041; Figure 12d) and non-basal (P<0.001; Figure 12e) disease subgroups, and in cats with a low Ki67 index (P=0.001; Figure 12f).

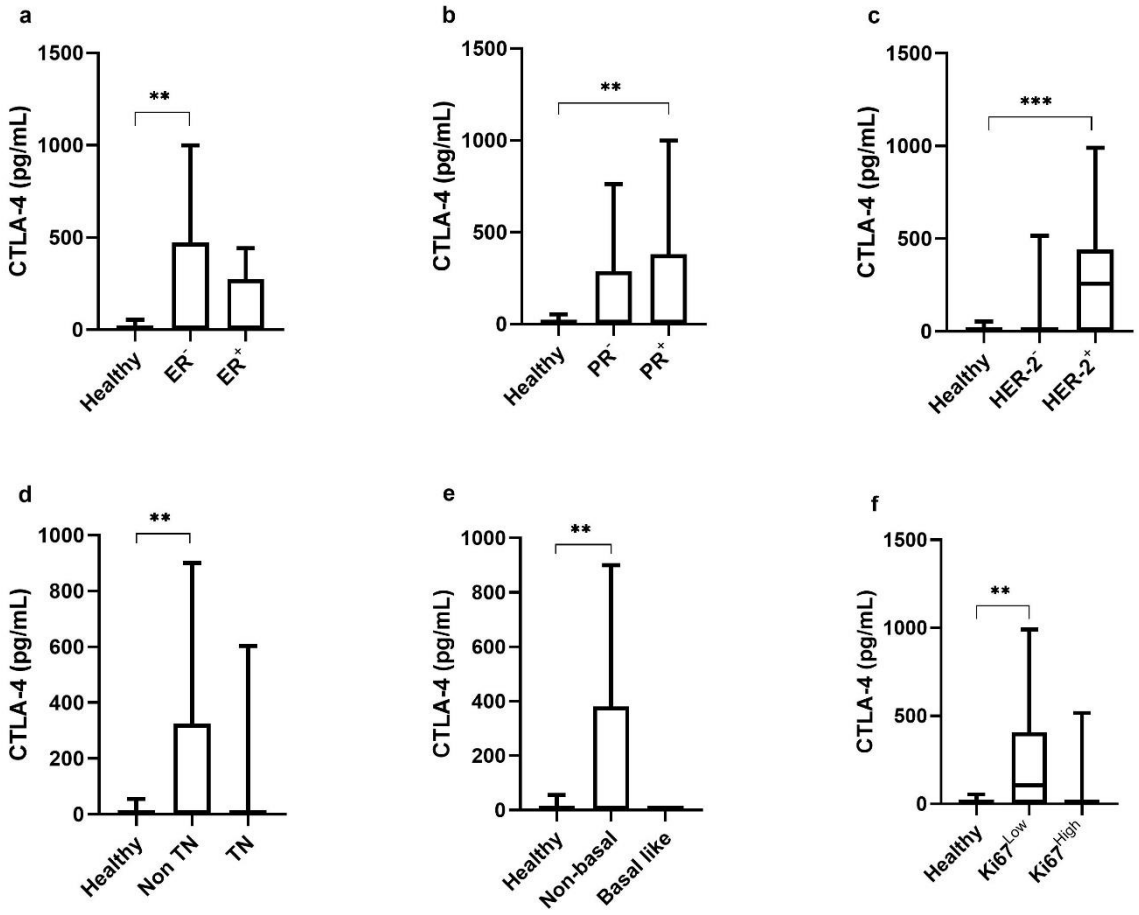

Figure 12. Box plot analysis of serum cytotoxic T-lymphocyte associated protein 4 (CTLA-4) levels and their association with immunohistochemical parameters: a) ER status; b) PR status; c) HER-2 status; d) Triple negative (TN) status; e) Basal status; f) Ki67 index (<14% or ≥14%). * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

2.3.2. Prognostic value of serum CTLA-4 for overall and disease-free survival

The detection limit (DL=31.3 pg/mL), lowest quartile (LQ=317.5 pg/mL), median (MD=459.4 pg/mL) and highest quartile (HQ=875.5 pg/mL) were used as cut-point values to stratify the data into high and low expression of the regulator. Groups were then tested to assess the significance of CTLA-4 levels in terms of OS and DSF. Kaplan-Meier survival analysis did not show any significant relationship between CTLA-4 levels and either OS or DFS for any of the four cut-point values tested (all P values were > 0.05; Figure 13).

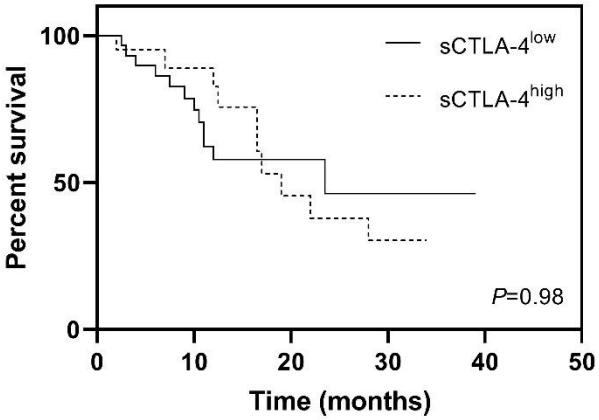

Figure 13. Kaplan–Meier survival curve for serum cytotoxic T-lymphocyte associated protein 4 (CTLA-4) high and low groups. The detection limit was used as cut-point.

Median survival times were greater in the CTLA-4^{low} group for three of the cut-point values tested (DL, LQ and MD). Median survival time was greater in the CTLA-4^{high} group when the highest quartile was used as a cut-point (Table 2).

Table 2. Median survival times (months) for cytotoxic T-lymphocyte associated protein 4 (CTLA-4) high and low groups. DL – Detection Limit; LQ – Lowest Quartile; MD – Median; HQ – Highest Quartile.

Cut-point	CTLA-4^{low}	CTLA-4^{high}
DL (31.3 pg/mL)	23.5	19
LQ (317.5 pg/mL)	23.5	22
MD (459.4 pg/mL)	23.5	17
HQ (875.5 pg/mL)	22	28

2.3.3. Serum TNF-α and IL-6 levels

TNF-α and IL-6 levels were detectable in all the 57 cats assessed, with medians of 36.10 pg/mL (range 19.11–463.5 pg/mL) and 65.94 pg/mL (range 39.17–766.5 pg/mL) respectively. The data were tested for associations with clinicopathological features with TNF-α levels in the cats with mammary carcinoma being significantly higher than those in the healthy group (P=0.011; Figure 14).

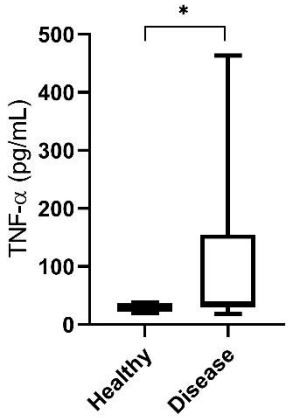

Figure 14. Box plot analysis of serum tumor necrosis factor alpha (TNF-α) levels in healthy cats and cats with mammary carcinoma. * *P* < 0.05; ** *P* < 0.001; *** *P* < 0.0001.

As with CTLA-4, TNF-α levels were significantly increased in cats with stage I tumors (*P*<0.001; Figure 15a), smaller tumors (*P*<0.001; Figure 15b) and no tumor necrosis (*P*=0.004; Figure 15c). However, there was no significant correlation between TNF-α levels and either age, lymph node involvement or lymphatic vessel invasion.

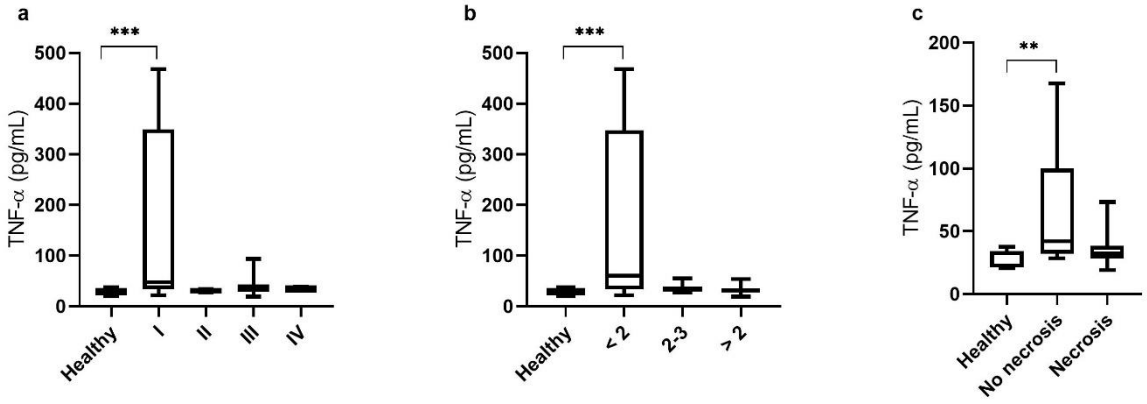

Figure 15. Box plot analysis of serum tumor necrosis factor alpha (TNF-α) levels and their association with clinicopathological parameters: a) clinical stage; b) tumor size (cm); c) tumor necrosis. * *P* < 0.05; ** *P* < 0.001; *** *P* < 0.0001.

TNF-α levels were also increased in cats with positive PR (*P*=0.004; Figure 16a), positive HER-2 (*P*=0.004; Figure 16b), non-TN (*P*=0.007; Figure 16c), and non-basal status (*P*=0.008; Figure 16d) and low Ki67 index (*P*=0.005; Figure 16e).

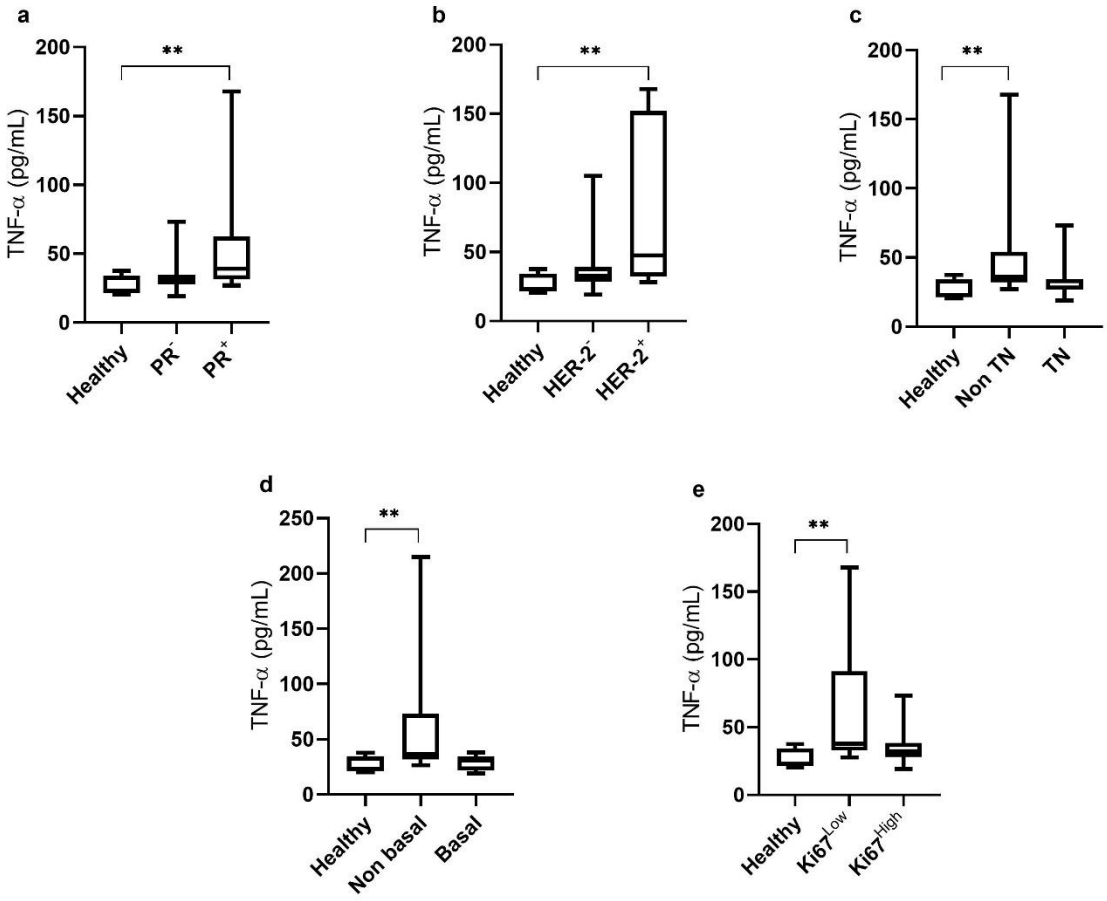

Figure 16. Box plot analysis of serum tumor necrosis factor alpha (TNF-α) levels and their association with immunohistochemical parameters: a) PR status; b) HER-2-status; c) Triple negative (TN) status; d) Basal status; e) Ki67 index (<14% or ≥ 14%). * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

In parallel, serum IL-6 levels in the cats with mammary carcinoma were significantly higher when compared with healthy cats (P=0.021; Figure 17).

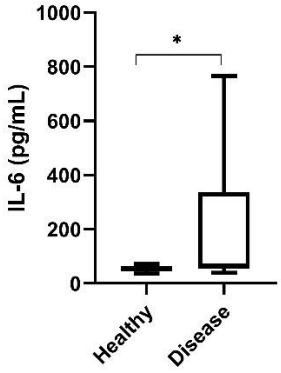

Figure 17. Box plot analysis of serum interleukin 6 (IL-6) levels in healthy cats and cats with mammary carcinoma. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

There was no significant relationship between IL-6 levels and either clinical stage, tumor size, tumor necrosis or lymphatic vessel invasion. However, serum IL-6 levels were significantly increased in older cats (*P*=0.030; Figure 18a) and cats with no lymph node involvement (*P*=0.021; Figure 18b).

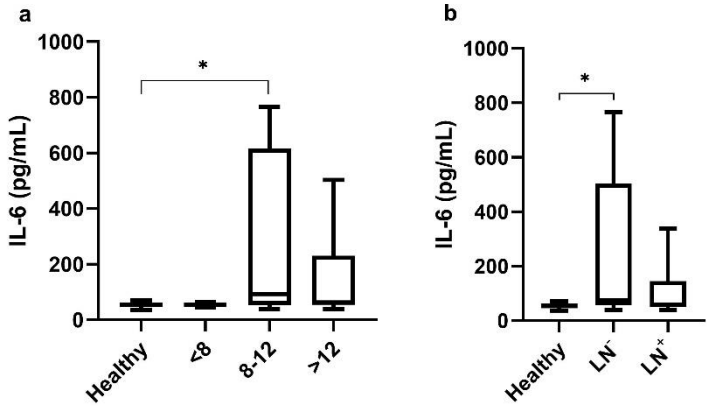

Figure 18. Box plot analysis of serum interleukin 6 (IL-6) levels and their association with clinicopathological criteria: a) age (years); b) lymph node status. * *P* < 0.05; ** *P* < 0.001; *** *P* < 0.0001.

Serum IL-6 levels were also higher in the cats with PR-positive (*P*=0.042; Figure 19a) and low Ki67 index (*P*=0.020; Figure 19b) mammary carcinoma.

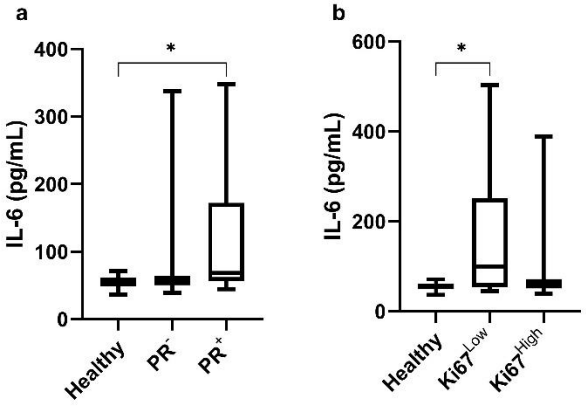

Figure 19. Box plot analysis of serum interleukin 6 (IL-6) levels and their association with immunohistochemical parameters: a) PR status; b) Ki67 index (<14% or ≥ 14%). * *P* < 0.05; ** *P* < 0.001; *** *P* < 0.0001.

2.3.4. Prognostic value of TNF-α and IL-6 for overall and disease-free survival

To date, there are no studies specifying the cut-off values for either TNF-α or IL-6 in predicting the prognosis of cats with mammary carcinoma. In our study, the Receiver operator curve (ROC) analysis of sensitivity versus specificity of the ELISA was performed to determine

the best cut-point values for predicting OS and DFS as well as predict the diagnostic value of these cytokines (Figure 20). The best cut-point values identified for TNF-α and IL-6 were 33.2 pg/mL and 58.4 pg/mL, with an area under the curve of 0.75 (*P*=0.006) and 0.71 (*P*=0.022) respectively.

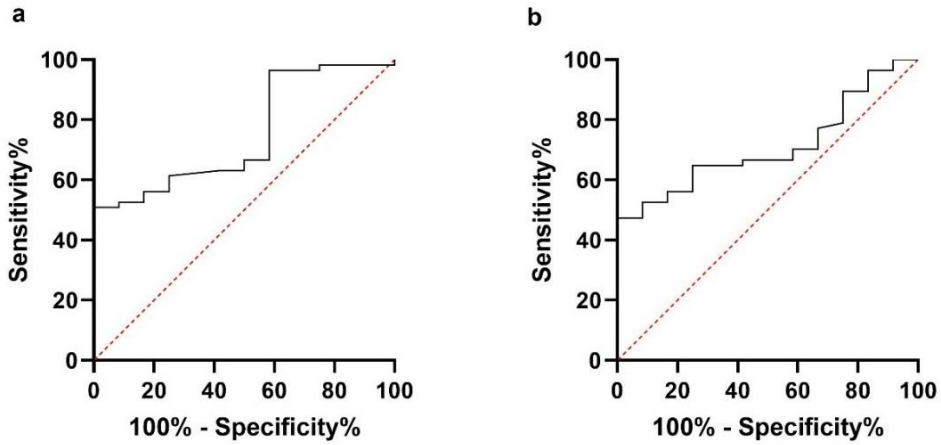

Figure 20. Receiver operator curve (ROC) analysis of sensitivity versus specificity for tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6): a) TNF-α AUC 0.75 (*P*=0.006); b) IL-6 AUC 0.71 (*P*=0.022).

In Kaplan–Meier survival analysis, no significant relationship was found between either TNF-α or IL-6 serum levels and survival (all *P* values were > 0.05). Survival analysis based on median (37.8 pg/mL and 65.94 pg/mL respectively; MD) and highest quartile values (146.2 pg/mL and 336.6 respectively; HQ) also did not show any statistical significance (all *P* values were > 0.05; Figure 21).

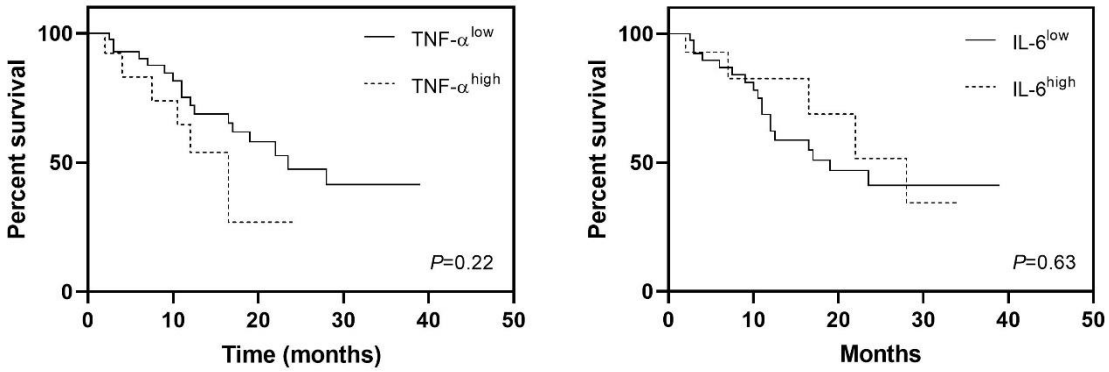

Figure 21. Kaplan–Meier survival curve for tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) high and low groups. The highest quartile is used as cut-point.

For TNF-α median survival time was greater in the TNF-α ^{high} group when the cut-point value obtained from the ROC curve analysis was used. Median survival times were greater in the TNF-α ^{low} group for the other two cut-point values tested (Table 3).

Table 3. Median survival times (months) for tumor necrosis factor alpha (TNF-α) high and low groups. ROC – Receiver Operating Curve; MD – Median; HQ – Highest Quartile.

Cut-point	TNF-α ^{low}	TNF-α ^{high}
ROC (33.2 pg/mL)	19	23.5
MD (38.7 pg/mL)	28	16.5
HQ (146.2 pg/mL)	23.5	16.5

We were unable to determine the median survival time for the IL-6 ^{low} group when the cut-point obtained from the ROC curve analysis was used. Survival time was greater in the IL-6 ^{low} group when the median was used as a cut-point. Median survival time was greater in the IL-6^{high} group when the highest quartile was used (Table 4).

Table 4. Median survival times (months) for interleukin 6 (IL-6) high and low groups. ROC – Receiver Operating Curve; MD – Median; HQ – Highest Quartile; UD – Undefined.

Cut-point	IL-6 ^{low}	IL-6 ^{high}
ROC (58.4 pg/mL)	UD	16.5
MD (65.9 pg/mL)	23.5	19
HQ (336.6 pg/mL)	19	28

2.3.5. Correlation between CTLA-4 and serum TNF-α and IL-6 levels

A significant positive correlation was found between sCTLA-4 and both TNF-α (R= 0.8860, P<0.001; Figure 22a) and IL-6 levels (R= 0.7285, P<0.001; Figure 22b). Serum TNF-α and IL-6 levels were also positively correlated (R= 0.7451, P<0.001; Figure 22c).

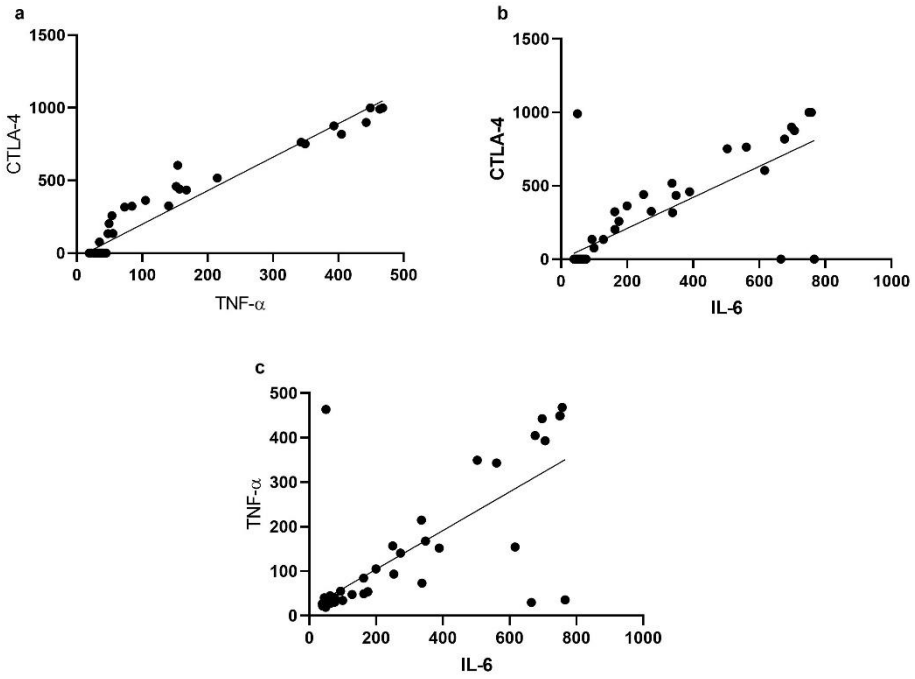

Figure 22. Pearson correlation of serum CTLA-4 and serum TNF-α and IL-6 levels: a) serum CTLA-4 and TNF-α (R= 0.8860, P<0.001); b) serum CTLA-4 and IL-6 (R= 0.7285, P<0.001); c) TNF-α and IL-6 (R= 0.7451, P<0.001).

2.4. Discussion

2.4.1. Serum CTLA-4 is increased in cats with mammary carcinoma and correlates with less aggressive clinicopathological features and positive HER-2 status

Various published data report increased serum levels of CTLA-4 in cancer patients (Erfani et al. 2010; Zhang et al. 2016; Liu, Xie, et al. 2017), with sCTLA-4 showing favourable prognostic significance in breast cancer (Liu, Hu, et al. 2017). Accordingly, our investigations revealed that CTLA-4 is elevated in the sera of cats with mammary carcinoma. Furthermore, higher serum CTLA-4 levels were correlated with less aggressive clinicopathological features: smaller tumors; lower stage; absence of necrosis, lymph node involvement or lymphatic vessel invasion; positive hormone receptor status; non-TN, non-basal status; and low Ki67 index. The elevated serum CTLA-4 levels may reflect an ongoing inflammatory response in the tumor microenvironment, where post-activated T-lymphocytes are actively expressing sCTLA-4. Proteolytic cleavage of mCTLA-4 concurrent with T-lymphocyte exhaustion, a progressive loss of effector function due to prolonged antigen stimulation, may also be a contributing factor (Sakthivel et al. 2010), as this event is also observed in cancer patients.

The association with less aggressive clinicopathological features may be explained by blockade of the mCTLA-4 pathway by sCTLA-4, leading to activation of effector T-lymphocytes which contribute to a tumor-suppressive immune environment, as described in humans (Saverino et al. 2007; Pérez-García et al. 2013; Simone et al. 2014). Another hypothesis is that CTLA-4 may be mediating negative signals into the tumor cells, thus leading to inhibition of tumor cell proliferation and/or induction of apoptotic cell death (Salvi et al. 2012).

In this context, the association found between elevated serum CTLA-4 and HER-2-positive tumors would seem paradoxical, as this subtype is frequently associated with more aggressive features and poorer prognosis in humans. However, a study on HER-2 expression in feline mammary carcinoma found a positive correlation between higher HER-2 mRNA expression and better clinical outcome (Santos et al. 2013), in accordance with our results. Furthermore, a study in a mouse model of HER-2 positive breast cancer found that induced intratumoral expression of a CTLA-4 monoclonal antibody not only failed to exert anti-tumor effects, but instead stimulated tumor growth and increased the percentage of immunosuppressive NK cells (Persson et al. 2011).

2.4.2. Higher serum CTLA-4 tends to correlate with improved survival in cats with mammary carcinoma

In veterinary oncology, particularly with cats, it is often difficult to follow the diseases as they progress. As such, most studies are retrospective, and lack significant data associated with survival (Zappulli et al. 2005). Although we did have access to survival data in this study, we were unable to prove a statistically significant association between serum CTLA-4 levels and either OS or DFS for the study group population. However, the crossing survival curves suggest that for some cats, serum CTLA-4 might have a positive prognostic value and/or that there is a time-dependent effect in play. In these situations, by evaluating the Kaplan–Meier curves for each level of the subgroup it's possible to identify if there is a qualitative interaction driving the curves to cross for the population as a whole (Barraclough et al. 2011). We were, unfortunately, unable to perform subgroup analyses because of the small sample size, so cannot confirm the existence of a subgroup effect.

Another possibility, when Kaplan–Meier curves come together, is that by this time the data are very mature because there are few patients still at risk as most have already died or been censored (Barraclough et al. 2011). Given the poor survival time reported for cats with mammary carcinoma this could very possibly be the case. If we then consider the data before the timepoint at which the survival curves cross as significant, the CTLA-4 high group seems to be associated with improved survival.

The higher median survival time observed for the CTLA-4^{high} group when the highest quartile was used as a cut-point (28 months vs 22 months for the CTLA-4^{low} group) also suggests a potentially protective role for serum CTLA-4, which may, however, be concentration dependent. These data go in accordance with the strong association found between serum CTLA-4 levels and lymph node involvement, lymphatic vessel invasion, and tumor size, which are considered some of the most reliable prognostic parameters for feline mammary carcinoma (Zappulli et al. 2015). Thus, it is our opinion that the potential value of serum CTLA-4 levels as a prognostic parameter for cats with mammary carcinoma should not be dismissed. Further investigation is required.

2.4.3. Serum TNF-α levels are increased in cats with mammary carcinoma and correlate with less aggressive clinicopathological features and positive HER-2 status

Most cytokines are overexpressed in breast cancer when compared with normal tissues, and their serum levels are increased (E. Goldberg and L. Schwertfeger 2010). TNF-α is an important pro-inflammatory cytokine with well-known cytotoxic effects. Studies have shown that TNF-α production is significantly related to HER-2 overexpression in some cancers (Melczer et al. 2003). However, activation of HER-2 suppresses the cytotoxic effects of TNF-α, being correlated with poor survival (Zhou et al. 2000). In our study, increased serum levels of TNF-α were found in cats with mammary carcinoma, particularly in HER-2 positive tumors, being correlated with less aggressive clinicopathological features.

Concerning this result, we propose that TNF-α may be contributing to the modulation of the tumor immune response through an alternative, indirect mechanism. It has been shown that TNF-α can upregulate CD86 and ICOSL expression (Sato et al. 1999; Rutella and Locatelli 2012). ICOS is an inducible T-lymphocyte co-stimulator structurally and functionally related to CD28 which enhances T-lymphocyte responses to antigens, namely proliferation, secretion of cytokines, upregulation of molecules that mediate cell to cell interactions, and effective help for antibody secretion by B cells (Hutloff et al. 1999).

As previously mentioned, CTLA-4 is expressed on the cell surface transiently and then recycled through endocytosis initiated by the attachment of adapter protein 2 (AP-2) on its cytoplasmic tail (Chuang et al. 1997). However, the interaction of AP-2 with CTLA-4 may be replaced by activated phosphatidylinositol 3-kinase (PI3K) (Chuang et al. 1997). Since surface CTLA-4 and ICOS are generally gathered around activated TCR, PI3K recruited by ICOS-ICOSL interaction (Gigoux et al. 2009) might compete with AP-2, reducing endocytosis of CTLA-4. A study demonstrating decreased surface CTLA-4 expression and reduced

suppressive capacity in Tregs caused by ICOS-Ig partly supports this hypothesis (Zheng et al. 2013).

If in our study population the higher levels of serum TNF-α were to be positively correlated with decreased tissue expression of CTLA-4, together with an upregulation of CD86, which has a relative preference for CD28 compared to CD80 (Pentcheva-Hoang et al. 2004; Esensten et al. 2016) we might assume that TNF-α is promoting CD8+ T-lymphocyte activation in these animals, contributing to a tumor-suppressive immune response consistent with less aggressive clinicopathological features. We are investigating CTLA-4 expression in the matched tumor tissue sections and hope to be able to determine if such an association exists.

2.4.4. Higher serum TNF-α tends to correlate with worse survival in cats with mammary carcinoma

Similarly to what was observed for CTLA-4, Kaplan-Meier survival analysis did not show a statistically significant association between serum TNF-α levels and either OS or DFS. The TNF-α^{high} group seems to tend towards worse survival, and this is supported by the lower median survival when the median (16.5 vs 28 months for the TNF-α^{low} group) and highest quartile (16.5 vs 23.5 months for the TNF-α^{low} group) are used as cut-points. When the cut-point obtained from ROC analysis is used, however, this trend is inverted with the TNF-α^{high} having a higher median survival time (23.5 vs 19 months for the TNF-α^{low} group). These data seem to suggest that while some serum TNF-α can be beneficial, higher concentrations eventually prove detrimental. Indeed, dose-dependent opposing effects of TNF-α have been previously reported (reviewed in Tse et al. 2012). We also cannot exclude a possible subgroup effect, associated, for example, with a specific molecular subtype such as HER-2 positive. As already stated, activation of HER-2 suppresses the cytotoxic effects of TNF-α via interference with the TNF-α apoptotic pathway, and this correlates with a worse prognosis (Zhou et al. 2000). It may also be that the elevated TNF-α serum levels are not directly contributing to prognosis and are just a reflection of advanced disease.

2.4.5. Serum IL-6 levels are increased in cats with mammary carcinoma and correlate with lymph-node negative status and a luminal A-like subtype

In this study higher serum levels of IL-6 were found in the cats with mammary carcinoma, when compared to healthy animals. This scenario may reflect a predominant infiltration of M1 TAMs and MDSCs, an event reported at the earlier stages of tumor development in humans and mouse, as these cells are known to be the main source of pro-inflammatory cytokines. Tumor derived IL-6 may also be a contributing factor.

We also found that increased serum IL-6 levels were correlated with a lymph-node negative status, a PR-positive status and a low Ki67 index. The progesterone receptor is an ER-regulated gene expressed in over two-thirds of ER-positive breast cancers, being more highly expressed in the luminal A subtype (Lim et al. 2016). According to the criteria proposed by the St. Gallen International Expert Consensus, the luminal A breast cancer subtype is defined as having positive ER and or PR, negative HER-2, low Ki67 index and any CK5/6 status (Goldhirsch et al. 2013). Luminal A tumors tend to respond well to endocrine therapy, have low recurrence scores and generally carry a good prognosis. In cats, a luminal A mammary carcinoma subtype has been identified and is also associated with improved survival (Soares, Correia, et al. 2016). Studies have demonstrated an association between high IL-6 expression and PR positivity (Danforth and Sgagias 1993). IL-6 down-regulates the ER and enhances estradiol stimulation of PR synthesis. Our findings expand this concept by indicating a potential association between increased serum IL-6 levels and a luminal-A like feline mammary tumor subtype.

2.4.6. Higher serum IL-6 tends to correlate with improved survival in cats with mammary carcinoma

Again, Kaplan-Meier survival analysis did not show a statistically significant association between serum IL-6 levels and either OS or DFS. The survival curves for the IL-6 high and low groups, however, cross at several time-points, suggesting a possible subgroup effect. The IL-6^{high} group seems to tend towards improved survival. Median survival time is higher for the IL-6^{high} group when the highest quartile is used as a cut-point (28 vs 19 months for the $IL6^{low}$ group) supporting this tendency. Taken together, these data seem to suggest a positive prognostic role for IL-6.

This cytokine has been known to skew TAM polarization to the tumor-suppressing M1 phenotype (Madeddu et al. 2018), and induce CD4+ T-lymphocyte differentiation into the Th17 phenotype (Wang et al. 2009). If we consider that both M1 TAMs and Th17 T-lymphocytes are capable of promoting anti-tumor immune responses, it is tempting to envision that the interaction between these cell types is balancing the tumor-promoting immune responses in the microenvironment, an event that would be consistent with our observations. The direct actions of IL-6 may also play an important role, since it can down-regulate the ER, antagonizing estradiol stimulation of tumor cell growth (Danforth and Sgagias 1993).

2.4.7. Serum CTLA-4 levels correlate with TNF-α and IL-6 levels in cats with mammary carcinoma

Our study revealed a positive correlation between CTLA-4 and the pro-inflammatory cytokine levels in the sera of cats with feline mammary carcinoma. Several studies indicate that serum CTLA-4 is associated with pro-inflammatory cytokine levels. Sakthivel and colleagues found that levels of sCTLA-4 were directly related to the levels of pro-inflammatory cytokines IL-6, IL-1α, IL-1β, TNF-α and IFN-γ on a study of the Scandinavian 70 year old population (Sakthivel et al. 2010). Interestingly, CTLA-4 and IL-6 serum levels were also correlated with age in our study, paralleling the findings of Sakthivel and colleagues. Another study, conducted on mice, also points to the association between sCTLA-4 and pro-inflammatory cytokine levels. Grohmann and colleagues found that CTLA-4-Ig can induce production of IFN-γ and TNF-α in DCs (Grohmann et al. 2002). A possible reason for this direct relation may be activation of certain cell types involved in cancer-associated inflammation, like Tregs, which are the main source of sCTLA-4 (Ward et al. 2013). Our ongoing investigations into FoxP3 expression in the serum matched tumor tissue sections could shed some light on the issue.

2.5. Conclusions

Despite some inherent challenges, cats are increasingly being used as models for the study of spontaneous human diseases. The epidemiological and histological similarities between feline mammary tumors and human breast cancer make them especially valuable in the field of comparative oncology and contribute a diverse range of opportunities to the “One Health” concept. To the best of our knowledge, the present study is the first to report the serum profiles of CTLA-4 and pro-inflammatory cytokines TNF-α and IL-6 in cats with mammary carcinoma and their correlation with clinicopathological features. The results presented clearly demonstrate that serum CTLA-4 levels are increased in cats with mammary carcinoma and are positively correlated with pro-inflammatory cytokines levels, advancing the concept of an immunomodulatory role for this regulator in breast cancer pathogenesis, as described for humans. Furthermore, we demonstrate a clear association between immune activation in the mammary tumor microenvironment, as evidenced by the higher levels of serum CTLA-4, IL-6 and TNF-α, and less aggressive clinicopathological features, consistent with previous research. Our findings expand this concept by indicating a potential association with specific breast cancer subtypes, namely, HER-2 positive and luminal-A. Although the results from the survival analysis were inconclusive, they suggest a potentially concentration-dependent protective role for serum CTLA-4 and IL-6, as evidenced by higher median survival times in

the CTLA-4^{high} and IL-6^{high} groups. In contrast, TNF-α seems to be a negative prognostic factor. An intriguing question that remains is how serum CTLA-4 influences or is influenced by the pro-inflammatory cytokines. We are investigating immunohistochemical CTLA-4 expression in mammary tumor stroma as a first step in answering this question. Assessment of T-lymphocyte subtypes, tumor infiltrating macrophage and myeloid derived suppressor cell profiles, are also important features to evaluate in future studies.

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ANNEX I – Results of the Statistical Analysis

Results for CTLA-4

Descriptive Statistics for healthy animals

Number of values	12
Minimum	00.0
25% Percentile	00.0
Median	00.0
75% Percentile	00.0
Maximum	54.8
Range	54.8
Mean	4.57
Std. Deviation	15.8
Std. Error of Mean	4.56

Descriptive Statistics for animals with disease (all)

Number of values	57
Minimum	0.0
25% Percentile	0.0
Median	0.0
75% Percentile	363.12
Maximum	999.3
Range	999.3
Mean	222.6
Std. Deviation	337.0
Std. Error of Mean	44.64

Descriptive Statistics for animals with disease (detectable)

Number of values	23
Minimum	77.05
25% Percentile	317.05
Median	459.4
75% Percentile	875.5
Maximum	999.3
Range	922.2
Mean	551.7
Std. Deviation	315.7
Std. Error of Mean	65.71

Mann-Whitney test of Healthy vs Disease

P value	.022
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	236.5 , 1655
Mann-Whitney U	170.5
Difference between medians	
Median of column A	0.000, n=11
Median of column B	0.000, n=50
Difference: Actual	0.000
Difference: Hodges-Lehmann	0.000

Kruskal-Wallis test of Age (years)

P value	.032
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	8.794
Data summary	
Number of treatments (columns)	4
Number of values (total)	64

Kruskal-Wallis test of Age (years): Multiple comparisons

Number of families	1			
Number of comparisons per family	6			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. <8	0.000	No	ns	>.999
Healthy vs. 8-12	-14.60	Yes	**	.009
Healthy vs. >12	-13.36	Yes	*	.023
<8 vs. 8-12	-14.60	No	ns	.127
<8 vs. >12	-13.36	No	ns	.170
8-12 vs. >12	1.246	No	ns	.783
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. <8	21.50	21.50	0.000	11
Healthy vs. 8-12	21.50	36.10	-14.60	11
Healthy vs. >12	21.50	34.86	-13.36	11
<8 vs. 8-12	21.50	36.10	-14.60	3
<8 vs. >12	21.50	34.86	-13.36	3
8-12 vs. >12	36.10	34.86	1.246	29

Kruskal-Wallis test of Stage

P value	.020
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	5
Kruskal-Wallis statistic	11.72
Data summary	
Number of treatments (columns)	5
Number of values (total)	61

Kruskal-Wallis test of Stage: Multiple comparisons

Number of families	1			
Number of comparisons per family	10			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. I	-17.87	Yes	**	.002
Healthy vs. II	-14.58	Yes	*	.049
Healthy vs. III	-8.615	No	ns	.100
Healthy vs. IV	0.000	No	ns	>.999
I vs. II	3.283	No	ns	.641
I vs. III	9.251	No	ns	.050
I vs. IV	17.87	No	ns	.053
II vs. III	5.968	No	ns	.366
II vs. IV	14.58	No	ns	.157
III vs. IV	8.615	No	ns	.332
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. I	21.50	39.37	-17.87	11
Healthy vs. II	21.50	36.08	-14.58	11
Healthy vs. III	21.50	30.12	-8.615	11
Healthy vs. IV	21.50	21.50	0.000	11
I vs. II	39.37	36.08	3.283	15
I vs. III	39.37	30.12	9.251	15
I vs. IV	39.37	21.50	17.87	15
II vs. III	36.08	30.12	5.968	6
II vs. IV	36.08	21.50	14.58	6
III vs. IV	30.12	21.50	8.615	26

Kruskal-Wallis test of Size (cm)

P value	<.001
Exact or approximate P value?	Approximate
P value summary	***
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	16.72

Data summary	
Number of treatments (columns)	4
Number of values (total)	59

Kruskal-Wallis test of Size (cm): Multiple comparisons

Number of families	1			
Number of comparisons per family	6			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. <2	-17.39	Yes	***	<.001
Healthy vs. 2-3	-10.47	Yes	*	.046
Healthy vs. >2	0.000	No	ns	>.999
<2 vs. 2-3	6.917	No	ns	.131
<2 vs. >2	17.39	Yes	***	<.001
2-3 vs. >2	10.47	Yes	*	.041
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. <2	21.50	38.89	-17.39	11
Healthy vs. 2-3	21.50	31.97	-10.47	11
Healthy vs. >2	21.50	21.50	0.000	11
<2 vs. 2-3	38.89	31.97	6.917	18
<2 vs. >2	38.89	21.50	17.39	18
2-3 vs. >2	31.97	21.50	10.47	18

Kruskal-Wallis test of Necrosis

P value	<.001
Exact or approximate P value?	Approximate
P value summary	***
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	20.14
Data summary	
Number of treatments (columns)	3
Number of values (total)	55

Kruskal-Wallis test of Necrosis: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. No necrosis	-18.68	Yes	***	<.001
Healthy vs. Necrosis	-3.200	No	ns	.447
No necrosis vs. Necrosis	15.48	Yes	***	<.001

Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. No necrosis	21.50	40.18	-18.68	11
Healthy vs. Necrosis	21.50	24.70	-3.200	11
No necrosis vs. Necrosis	40.18	24.70	15.48	14

Kruskal-Wallis test of Lymphatic vessel invasion (LVI)

P value	.007
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	9.999
Data summary	
Number of treatments (columns)	3
Number of values (total)	63

Kruskal-Wallis test of Lymphatic vessel invasion (LVI): Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. No LI	-14.07	Yes	**	.006
Healthy vs. LI	0.000	No	ns	>.999
No LI vs. LI	14.07	No	ns	.052
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. No LI	21.50	35.57	-14.07	11
Healthy vs. LI	21.50	21.50	0.000	11
No LI vs. LI	35.57	21.50	14.07	47

Kruskal-Wallis test of Lymph node status (LN)

P value	.021
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	7.695
Data summary	
Number of treatments (columns)	3
Number of values (total)	60

Kruskal-Wallis test of Lymph node status (LN): Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. LN-	-13.40	Yes	**	.007
Healthy vs. LN+	-7.214	No	ns	.214
LN- vs. LN+	6.186	No	ns	.175
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. LN-	21.00	34.40	-13.40	11
Healthy vs. LN+	21.00	28.21	-7.214	11
LN- vs. LN+	34.40	28.21	6.186	35

Kruskal-Wallis test of ER status

P value	.032
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	6.894
Data summary	
Number of treatments (columns)	3
Number of values (total)	63

Kruskal-Wallis test of ER Status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. ER-	-13.79	Yes	**	.009
Healthy vs. ER+	-9.821	No	ns	.113
ER- vs. ER+	3.968	No	ns	.409
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. ER-	21.50	35.29	-13.79	11
Healthy vs. ER+	21.50	31.32	-9.821	11
ER- vs. ER+	35.29	31.32	3.968	38

Kruskal-Wallis test of PR status

P value	.027

Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	7.216
Data summary	
Number of treatments (columns)	3
Number of values (total)	62

Kruskal-Wallis test of PR status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. PR-	-9.760	No	ns	.072
Healthy vs. PR+	-14.46	Yes	**	.007
PR- vs. PR+	-4.702	No	ns	.262
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. PR-	21.50	31.26	-9.760	11
Healthy vs. PR+	21.50	35.96	-14.46	11
PR- vs. PR+	31.26	35.96	-4.702	25

Kruskal-Wallis test of HER-2 status

P value	.001
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	13.60
Data summary	
Number of treatments (columns)	3
Number of values (total)	56

Kruskal-Wallis test of HER-2 status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. HER-2-	-5.515	No	ns	.200
Healthy vs. HER-2+	-18.59	Yes	***	<.001

HER-2- vs. HER-2+	-13.08	Yes	**	.002
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. HER-2-	21.50	27.01	-5.515	11
Healthy vs. HER-2+	21.50	40.09	-18.59	11
HER-2- vs. HER-2+	27.01	40.09	-13.08	34

Kruskal-Wallis test of Triple negative (TN) status

P value	.022
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	7.621
Data summary	
Number of treatments (columns)	3
Number of values (total)	59

Kruskal-Wallis test of Triple negative (TN) status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Non TN	-12.24	Yes	**	.010
Healthy vs. TN	-5.083	No	ns	.375
Non TN vs. TN	7.153	No	ns	.118
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Non TN	21.50	33.74	-12.24	11
Healthy vs. TN	21.50	26.58	-5.083	11
Non TN vs. TN	33.74	26.58	7.153	36

Kruskal-Wallis test of Basal status

P value	.004
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	11.17
Data summary	
Number of treatments (columns)	3
Number of values (total)	60

Kruskal-Wallis test of Basal status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Non-basal	-13.57	Yes	**	.005
Healthy vs. Basal	0.000	No	ns	>.999
Non-basal vs. Basal	13.57	Yes	*	.021
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Non-basal	21.00	34.57	-13.57	11
Healthy vs. Basal	21.00	21.00	0.000	11
Non-basal vs. Basal	34.57	21.00	13.57	42

Kruskal-Wallis test of Ki67 index (<14% or ≥ 14%)

P value	.003
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	11.50
Data summary	
Number of treatments (columns)	3
Number of values (total)	57

Kruskal-Wallis test of Ki67 index (<14% or ≥ 14%): Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Ki67Low	-16.09	Yes	**	.001
Healthy vs. Ki67High	-5.667	No	ns	.211
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Ki67Low	21.50	37.59	-16.09	11
Healthy vs. Ki67High	21.50	27.17	-5.667	11

Kaplan-Meyer Analysis of Overall Survival with Limit of detection (LD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.0004321	
df	1	
P value	0.9834	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.4171	
df	1	
P value	0.5184	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	23.50	
CTLA-4high	19.00	
Ratio (and its reciprocal)	1.237	0.8085
95% CI of ratio	0.5344 to 2.863	0.3493 to 1.871
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.9910	1.009
95% CI of ratio	0.4232 to 2.321	0.4309 to 2.363
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.9913	1.009
95% CI of ratio	0.4281 to 2.29	0.4357 to 2.336

Kaplan-Meyer Analysis of Overall Survival with Lowest quartile (LQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.07767	
df	1	
P value	.780	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.5115	
df	1	
P value	.474	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	23.50	
CTLA-4high	22.00	
Ratio (and its reciprocal)	1.068	0.9362
95% CI of ratio	0.4355 to 2.620	0.3817 to 2.296
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	1.135	0.8811
95% CI of ratio	0.4659 to 2.765	0.3617 to 2.146
Hazard Ratio (logrank)	A/B	B/A

Ratio (and its reciprocal)	1.134	0.8818
95% CI of ratio	0.4711 to 2.730	0.3663 to 2.123

Kaplan-Meyer Analysis of Overall Survival with Median (MD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.3226	
df	1	
P value	.570	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.004164	
df	1	
P value	.949	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	23.50	
CTLA-4high	17.00	
Ratio (and its reciprocal)	1.382	0.7234
95% CI of ratio	0.5409 to 3.533	0.2831 to 1.849
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.7458	1.341
95% CI of ratio	0.2711 to 2.052	0.4874 to 3.689
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.7641	1.309
95% CI of ratio	0.2799 to 2.086	0.4794 to 3.573

Kaplan-Meyer Analysis of Overall Survival with Highest quartile (HQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.6668	
df	1	
P value	.414	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.8603	
df	1	
P value	.354	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	22.00	
CTLA-4high	28.00	
Ratio (and its reciprocal)	0.7857	1.273

95% CI of ratio	0.2325 to 2.655	0.3766 to 4.301
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.5376	1.860
95% CI of ratio	0.1212 to 2.384	0.4194 to 8.249
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.6075	1.646
95% CI of ratio	0.1385 to 2.665	0.3752 to 7.222

Kaplan-Meyer Analysis of Disease Free Survival with Limit of detection (LD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.2623	
df	1	
P value	.609	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.03684	
df	1	
P value	.848	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	Undefined	
CTLA-4high	12.00	
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.7735	1.293
95% CI of ratio	0.2895 to 2.067	0.4838 to 3.454
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.7834	1.276
95% CI of ratio	0.2981 to 2.059	0.4856 to 3.355

Kaplan-Meyer Analysis of Disease Free Survival with Lowest quartile (LQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.001721	
df	1	
P value	.967	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.3606	
df	1	
P value	.548	

P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	16.00	
CTLA-4high	19.00	
Ratio (and its reciprocal)	0.8421	1.188
95% CI of ratio	0.2967 to 2.390	0.4183 to 3.371
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	1.023	0.9779
95% CI of ratio	0.3554 to 2.942	0.3399 to 2.814
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	1.022	0.9785
95% CI of ratio	0.3617 to 2.888	0.3463 to 2.765

Kaplan-Meyer Analysis of Disease Free Survival with Median (MD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.5497	
df	1	
P value	.458	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.09730	
df	1	
P value	.755	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	19.00	
CTLA-4high	12.00	
Ratio (and its reciprocal)	1.583	0.6316
95% CI of ratio	0.5163 to 4.856	0.2059 to 1.937
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.6097	1.640
95% CI of ratio	0.1648 to 2.255	0.4434 to 6.067
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.6654	1.503
95% CI of ratio	0.1877 to 2.359	0.4239 to 5.329

Kaplan-Meyer Analysis of Disease Free Survival with Highest quartile (HQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.02033	

df	1	
P value	.887	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.001370	
df	1	
P value	.970	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
CTLA-4low	16.00	
CTLA-4high	10.50	
Ratio (and its reciprocal)	1.524	0.6563
95% CI of ratio	0.2021 to 11.49	0.08703 to 4.949
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.8522	1.173
95% CI of ratio	0.09462 to 7.676	0.1303 to 10.57
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.8660	1.155
95% CI of ratio	0.1005 to 7.463	0.1340 to 9.953

Results for TNF-α

Descriptive Statistics for healthy animals

Number of values	12
Minimum	20.36
25% Percentile	21.45
Median	32.79
75% Percentile	34.22
Maximum	37.5
Range	17.15
Mean	28.28
Std. Deviation	6.61
Std. Error of Mean	1.9

Descriptive Statistics for animals with disease (all)

Number of values	57
Minimum	19.11
25% Percentile	30.76
Median	38.07
75% Percentile	146.2
Maximum	468.0
Range	448.9
Mean	112.3
Std. Deviation	141.2

Std. Error of Mean	44.64

Mann-Whitney test of Healthy vs Disease

P value	.011
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	224 , 1487
Mann-Whitney U	146
Difference between medians	
Median of column A	32.79, n=12
Median of column B	36.10, n=46
Difference: Actual	3.308
Difference: Hodges-Lehmann	10.63

Kruskal-Wallis test of Stage

P value	.005
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	5
Kruskal-Wallis statistic	15.01
Data summary	
Number of treatments (columns)	5
Number of values (total)	59

Kruskal-Wallis test of Stage: Multiple comparisons

Number of families	1			
Number of comparisons per family	10			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. I	-23.13	Yes	***	<.001
Healthy vs. II	-0.5333	No	ns	.953
Healthy vs. III	-7.021	No	ns	.247
Healthy vs. IV	-10.83	No	ns	.328
I vs. II	22.60	Yes	*	.011
I vs. III	16.11	Yes	**	.004
I vs. IV	12.30	No	ns	.257
II vs. III	-6.488	No	ns	.442
II vs. IV	-10.30	No	ns	.411
III vs. IV	-3.813	No	ns	.717
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. I	20.67	43.80	-23.13	12
Healthy vs. II	20.67	21.20	-0.5333	12

Healthy vs. III	20.67	27.69	-7.021	12
Healthy vs. IV	20.67	31.50	-10.83	12
I vs. II	43.80	21.20	22.60	15
I vs. III	43.80	27.69	16.11	15
I vs. IV	43.80	31.50	12.30	15
II vs. III	21.20	27.69	-6.488	5
II vs. IV	21.20	31.50	-10.30	5
III vs. IV	27.69	31.50	-3.813	24

Kruskal-Wallis test of Size (cm)

P value	.001
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	15.71
Data summary	
Number of treatments (columns)	4
Number of values (total)	60

Kruskal-Wallis test of Size (cm): Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. < 2 cm	-22.03	Yes	***	<.001
Healthy vs. 2-3 cm	-6.967	No	ns	.303
Healthy vs. > 2 cm	-3.449	No	ns	.622
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. < 2 cm	20.67	42.70	-22.03	12
Healthy vs. 2-3 cm	20.67	27.63	-6.967	12
Healthy vs. > 2 cm	20.67	24.12	-3.449	12

Kruskal-Wallis test of Necrosis

P value	.011
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	8.966
Data summary	
Number of treatments (columns)	3
Number of values (total)	55

Kruskal-Wallis test of Necrosis: Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. No necrosis	-18.79	Yes	**	.004
Healthy vs. Necrosis	-5.737	No	ns	.292
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. No necrosis	20.67	39.46	-18.79	12
Healthy vs. Necrosis	20.67	26.40	-5.737	12

Kruskal-Wallis test of PR status

P value	.004
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	11.07
Data summary	
Number of treatments (columns)	3
Number of values (total)	56

Kruskal-Wallis test of PR status: Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. PR-	-3.220	No	ns	.582
Healthy vs. PR+	-16.72	Yes	**	.004
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. PR-	20.67	23.89	-3.220	12
Healthy vs. PR+	20.67	37.39	-16.72	12

Kruskal-Wallis test of HER-2 status

P value	.013
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3

Kruskal-Wallis statistic	8.652
Data summary	
Number of treatments (columns)	3
Number of values (total)	56

Kruskal-Wallis test of HER-2 status: Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. HER2-	-7.674	No	ns	.163
Healthy vs. HER2+	-19.83	Yes	**	.004
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. HER2-	20.08	27.76	-7.674	12
Healthy vs. HER2+	20.08	39.91	-19.83	12

Kruskal-Wallis test of Triple negative (TN) status

P value	.004
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	11.07
Data summary	
Number of treatments (columns)	3
Number of values (total)	57

Kruskal-Wallis test of Triple negative (TN) status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Non TN	-14.93	Yes	**	.007
Healthy vs. TN	-0.05303	No	ns	.994
Non TN vs. TN	14.88	Yes	**	.010
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Non TN	20.08	35.01	-14.93	12
Healthy vs. TN	20.08	20.14	-0.05303	12
Non TN vs. TN	35.01	20.14	14.88	34

Kruskal-Wallis test of Basal status

P value	.007
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	10.02
Data summary	
Number of treatments (columns)	3
Number of values (total)	58

Kruskal-Wallis test of Basal status: Multiple comparisons

Number of families	1			
Number of comparisons per family	3			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Non-basal	-14.90	Yes	**	.008
Healthy vs. Basal	0.1429	No	ns	.986
Non-basal vs. Basal	15.04	Yes	*	.030
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Non-basal	19.50	34.40	-14.90	12
Healthy vs. Basal	19.50	19.36	0.1429	12
Non-basal vs. Basal	34.40	19.36	15.04	39

Kruskal-Wallis test of Ki67 index (<14% or ≥ 14%)

P value	.012
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	8.852
Data summary	
Number of treatments (columns)	3
Number of values (total)	55

Kruskal-Wallis test of Ki67 index (<14% or ≥ 14%): Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Ki67Low	-17.34	Yes	**	.005

Healthy vs. Ki67High	-6.019	No	ns	.279
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Ki67Low	20.00	37.34	-17.34	12
Healthy vs. Ki67High	20.00	26.02	-6.019	12

ROC Analysis

Area	0.7515
Std. Error	0.06839
95% confidence interval	0.6174 to 0.8855
P value	.006
Data	
Controls	12
Patients	57
Missing Controls	0
Missing Patients	0

ROC Analysis: Sensitivity & Specificity

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 19.73	98.25	90.71% to 99.91%	0.000	0.000% to 24.25%	0.9825
> 20.48	98.25	90.71% to 99.91%	8.333	0.4274% to 35.39%	1.072
> 20.73	98.25	90.71% to 99.91%	16.67	2.961% to 44.80%	1.179
> 21.31	98.25	90.71% to 99.91%	25.00	8.894% to 53.23%	1.310
> 22.51	96.49	88.08% to 99.38%	25.00	8.894% to 53.23%	1.287
> 24.41	96.49	88.08% to 99.38%	33.33	13.81% to 60.94%	1.447
> 26.04	96.49	88.08% to 99.38%	41.67	19.33% to 68.05%	1.654
> 26.78	94.74	85.63% to 98.57%	41.67	19.33% to 68.05%	1.624
> 27.21	92.98	83.30% to 97.24%	41.67	19.33% to 68.05%	1.594
> 27.48	91.23	81.06% to 96.19%	41.67	19.33% to 68.05%	1.564
> 27.86	89.47	78.88% to 95.09%	41.67	19.33% to 68.05%	1.534
> 28.39	84.21	72.64% to 91.46%	41.67	19.33% to 68.05%	1.444
> 28.77	82.46	70.63% to 90.18%	41.67	19.33% to 68.05%	1.414
> 29.03	80.70	68.66% to 88.87%	41.67	19.33% to 68.05%	1.383
> 29.57	78.95	66.71% to 87.53%	41.67	19.33% to 68.05%	1.353
> 30.33	77.19	64.79% to 86.16%	41.67	19.33% to 68.05%	1.323
> 30.76	75.44	62.90% to 84.77%	41.67	19.33% to 68.05%	1.293
> 31.25	73.68	61.02% to 83.35%	41.67	19.33% to 68.05%	1.263
> 31.93	71.93	59.17% to 81.92%	41.67	19.33% to 68.05%	1.233
> 32.33	66.67	53.72% to 77.51%	41.67	19.33% to 68.05%	1.143
> 32.59	66.67	53.72% to 77.51%	50.00	25.38% to 74.62%	1.333
> 32.89	63.16	50.18% to 74.48%	50.00	25.38% to 74.62%	1.263
> 33.20	**63.16**	**50.18% to 74.48%**	**58.33**	**31.95% to 80.67%**	**1.516**
> 33.68	61.40	48.43% to 72.94%	75.00	46.77% to 91.11%	2.456
> 34.29	59.65	46.70% to 71.38%	75.00	46.77% to 91.11%	2.386
> 34.52	56.14	43.28% to 68.23%	75.00	46.77% to 91.11%	2.246
> 34.83	56.14	43.28% to 68.23%	83.33	55.20% to 97.04%	3.368
> 35.28	54.39	41.59% to 66.63%	83.33	55.20% to 97.04%	3.263
> 35.98	52.63	39.92% to 65.01%	83.33	55.20% to 97.04%	3.158
> 36.64	52.63	39.92% to 65.01%	91.67	64.61% to 99.57%	6.316
> 37.14	50.88	38.26% to 63.38%	91.67	64.61% to 99.57%	6.105
> 37.79	50.88	38.26% to 63.38%	100.0	75.75% to 100.0%	

> 38.28	49.12	36.62% to 61.74%	100.0	75.75% to 100.0%	
> 39.33	47.37	34.99% to 60.08%	100.0	75.75% to 100.0%	
> 42.51	42.11	30.19% to 55.02%	100.0	75.75% to 100.0%	
> 46.19	40.35	28.62% to 53.30%	100.0	75.75% to 100.0%	
> 48.43	38.60	27.06% to 51.57%	100.0	75.75% to 100.0%	
> 51.57	36.84	25.52% to 49.82%	100.0	75.75% to 100.0%	
> 54.49	35.09	24.00% to 48.06%	100.0	75.75% to 100.0%	
> 64.17	33.33	22.49% to 46.28%	100.0	75.75% to 100.0%	
> 78.70	31.58	21.00% to 44.48%	100.0	75.75% to 100.0%	
> 88.88	29.82	19.53% to 42.66%	100.0	75.75% to 100.0%	
> 99.30	28.07	18.08% to 40.83%	100.0	75.75% to 100.0%	
> 122.8	26.32	16.65% to 38.98%	100.0	75.75% to 100.0%	
> 146.2	24.56	15.23% to 37.10%	100.0	75.75% to 100.0%	
> 153.2	22.81	13.84% to 35.21%	100.0	75.75% to 100.0%	
> 155.7	21.05	12.47% to 33.29%	100.0	75.75% to 100.0%	
> 162.4	19.30	11.13% to 31.34%	100.0	75.75% to 100.0%	
> 191.3	17.54	9.819% to 29.37%	100.0	75.75% to 100.0%	
> 279.0	15.79	8.536% to 27.36%	100.0	75.75% to 100.0%	
> 346.3	14.04	7.287% to 25.32%	100.0	75.75% to 100.0%	
> 371.3	12.28	6.078% to 23.25%	100.0	75.75% to 100.0%	
> 399.0	10.53	4.914% to 21.12%	100.0	75.75% to 100.0%	
> 423.8	8.772	3.805% to 18.94%	100.0	75.75% to 100.0%	
> 445.9	7.018	2.763% to 16.70%	100.0	75.75% to 100.0%	
> 449.2	5.263	1.435% to 14.37%	100.0	75.75% to 100.0%	
> 456.3	3.509	0.6234% to 11.92%	100.0	75.75% to 100.0%	
> 465.7	1.754	0.08999% to 9.291%	100.0	75.75% to 100.0%	

Kaplan-Meyer Analysis of Overall Survival with value obtained from ROC analysis as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.2927	
df	1	
P value	.588	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.009108	
df	1	
P value	.924	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
TNF-a low	19.00	
TNF-a high	23.50	
Ratio (and its reciprocal)	0.8085	1.237
95% CI of ratio	0.3568 to 1.832	0.5458 to 2.803
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	1.260	0.7936
95% CI of ratio	0.5455 to 2.911	0.3436 to 1.833
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	1.249	0.8004
95% CI of ratio	0.5463 to 2.857	0.3500 to 1.830

Kaplan-Meyer Analysis of Overall Survival with Median (MD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	1.230	
df	1	
P value	.267	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	1.797	
df	1	
P value	.180	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
TNF-a low	28.00	
TNF-a high	16.50	
Ratio (and its reciprocal)	1.697	0.5893
95% CI of ratio	0.7488 to 3.846	0.2600 to 1.336
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.6197	1.614
95% CI of ratio	0.2660 to 1.444	0.6926 to 3.760
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.6370	1.570
95% CI of ratio	0.2775 to 1.462	0.6839 to 3.604

Kaplan-Meyer Analysis of Overall Survival with Highest quartile (HQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	1.470	
df	1	
P value	.225	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	1.480	
df	1	
P value	.224	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
TNF-a low	23.50	
TNF-a high	16.50	
Ratio (and its reciprocal)	1.424	0.7021
95% CI of ratio	0.5615 to 3.612	0.2768 to 1.781
Hazard Ratio (Mantel-Haenszel)	A/B	B/A

Ratio (and its reciprocal)	0.5007	1.997
95% CI of ratio	0.1636 to 1.532	0.6528 to 6.111
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.5740	1.742
95% CI of ratio	0.1926 to 1.711	0.5846 to 5.192

Kaplan-Meyer Analysis of Disease Free Survival with value obtained from ROC analysis as cut-point

Log-rank (Mantel-Cox) test		
Chi square	2.302	
df	1	
P value	.129	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	1.828	
df	1	
P value	.176	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
TNF-a low	12.00	
TNF-a high	Undefined	
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	2.188	0.4571
95% CI of ratio	0.7957 to 6.015	0.1663 to 1.257
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	2.031	0.4924
95% CI of ratio	0.7528 to 5.480	0.1825 to 1.328

Kaplan-Meyer Analysis of Disease Free Survival with Median (MD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.0003258	
df	1	
P value	.986	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.006315	
df	1	
P value	.937	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		

TNF-a low	19.00	
TNF-a high	16.00	
Ratio (and its reciprocal)	1.188	0.8421
95% CI of ratio	0.4582 to 3.078	0.3249 to 2.183
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	1.009	0.9910
95% CI of ratio	0.3801 to 2.679	0.3733 to 2.631
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	1.009	0.9915
95% CI of ratio	0.3892 to 2.614	0.3826 to 2.569

Kaplan-Meyer Analysis of Disease Free Survival with Highest quartile (HQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.05285	
df	1	
P value	.818	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.5120	
df	1	
P value	.474	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
TNF-a low	19.00	
TNF-a high	Undefined	
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	1.157	0.8645
95% CI of ratio	0.3342 to 4.004	0.2498 to 2.992
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	1.151	0.8690
95% CI of ratio	0.3489 to 3.795	0.2635 to 2.866

Results for IL-6

Descriptive Statistics for healthy animals

Number of values	12
Minimum	36.09
25% Percentile	48.98
Median	56.48
75% Percentile	61.58
Maximum	71.07
Range	34.98

Mean	55.23
Std. Deviation	9.43
Std. Error of Mean	2.72

Descriptive Statistics for animals with disease (all)

Number of values	57
Minimum	39.17
25% Percentile	54.36
Median	65.94
75% Percentile	336.6
Maximum	766.5
Range	727.3
Mean	218.4
Std. Deviation	245.1
Std. Error of Mean	32.46

Mann-Whitney test of Healthy vs Disease

P value	.021
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	275.5 , 2140
Mann-Whitney U	197.5
Difference between medians	
Median of column A	56.48, n=12
Median of column B	65.94, n=57
Difference: Actual	9.457
Difference: Hodges-Lehmann	17.99

Kruskal-Wallis test of Age (years)

P value	.030
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	8.945
Data summary	
Number of treatments (columns)	4
Number of values (total)	67

Kruskal-Wallis test of Age (years): Multiple comparisons

Number of families	1			
Number of comparisons per family	6			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. <8	2.333	No	ns	.836
Healthy vs. 8-12	-17.30	Yes	**	.009
Healthy vs. >12	-10.64	No	ns	.135
<8 vs. 8-12	-19.63	No	ns	.058
<8 vs. >12	-12.98	No	ns	.224
8-12 vs. >12	6.658	No	ns	.233
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. <8	22.96	20.63	2.333	12
Healthy vs. 8-12	22.96	40.26	-17.30	12
Healthy vs. >12	22.96	33.60	-10.64	12
<8 vs. 8-12	20.63	40.26	-19.63	4
<8 vs. >12	20.63	33.60	-12.98	4
8-12 vs. >12	40.26	33.60	6.658	31

Kruskal-Wallis test of Lymph node status (LN)

P value	.021
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	7.714
Data summary	
Number of treatments (columns)	3
Number of values (total)	63

Kruskal-Wallis test of Lymph node status (LN): Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. LN-	-15.40	Yes	*	.012
Healthy vs. LN+	-5.188	No	ns	.459
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. LN-	22.13	37.53	-15.40	12
Healthy vs. LN+	22.13	27.31	-5.188	12

Kruskal-Wallis test of PR status

P value	.042
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	6.363
Data summary	
Number of treatments (columns)	3
Number of values (total)	57

Kruskal-Wallis test of PR status: Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. PR-	-2.607	No	ns	.659
Healthy vs. PR+	-12.93	Yes	*	.030
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. PR-	22.96	25.57	-2.607	12
Healthy vs. PR+	22.96	35.89	-12.93	12

Kruskal-Wallis test of Ki67 index (<14% or ≥ 14%)

P value	.048
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	3
Kruskal-Wallis statistic	6.072
Data summary	
Number of treatments (columns)	3
Number of values (total)	59

Kruskal-Wallis test of Ki67 index (<14% or ≥ 14%): Multiple comparisons

Number of families	1			
Number of comparisons per family	2			
Alpha	0.05			
Uncorrected Dunn's test	Mean rank diff.	Significant?	Summary	Individual P Value
Healthy vs. Ki67Low	-15.10	Yes	*	.020
Healthy vs. Ki67High	-5.292	No	ns	.367
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
Healthy vs. Ki67Low	22.96	38.06	-15.10	12
Healthy vs. Ki67High	22.96	28.25	-5.292	12

ROC Analysis

Area	0.7113
Std. Error	0.06547
95% confidence interval	0.5829 to 0.8396
P value	.022
Data	
Controls	12
Patients	57
Missing Controls	0
Missing Patients	0

ROC Analysis: Sensitivity & Specificity

	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 37.63	100.0	93.69% to 100.0%	8.333	0.4274% to 35.39%	1.091
> 39.45	98.25	90.71% to 99.91%	8.333	0.4274% to 35.39%	1.072
> 41.41	96.49	88.08% to 99.38%	8.333	0.4274% to 35.39%	1.053
> 43.84	96.49	88.08% to 99.38%	16.67	2.961% to 44.80%	1.158
> 45.25	92.98	83.30% to 97.24%	16.67	2.961% to 44.80%	1.116
> 46.61	91.23	81.06% to 96.19%	16.67	2.961% to 44.80%	1.095
> 47.53	89.47	78.88% to 95.09%	16.67	2.961% to 44.80%	1.074
> 48.46	89.47	78.88% to 95.09%	25.00	8.894% to 53.23%	1.193
> 49.47	87.72	76.75% to 93.92%	25.00	8.894% to 53.23%	1.170
> 50.09	85.96	74.68% to 92.71%	25.00	8.894% to 53.23%	1.146
> 50.72	84.21	72.64% to 91.46%	25.00	8.894% to 53.23%	1.123
> 51.18	82.46	70.63% to 90.18%	25.00	8.894% to 53.23%	1.099
> 51.49	80.70	68.66% to 88.87%	25.00	8.894% to 53.23%	1.076
> 52.15	78.95	66.71% to 87.53%	25.00	8.894% to 53.23%	1.053
> 53.08	77.19	64.79% to 86.16%	33.33	13.81% to 60.94%	1.158
> 54.36	75.44	62.90% to 84.77%	33.33	13.81% to 60.94%	1.132
> 55.24	73.68	61.02% to 83.35%	33.33	13.81% to 60.94%	1.105
> 55.57	71.93	59.17% to 81.92%	33.33	13.81% to 60.94%	1.079
> 55.96	70.18	57.34% to 80.47%	33.33	13.81% to 60.94%	1.053
> 56.25	70.18	57.34% to 80.47%	41.67	19.33% to 68.05%	1.203
> 56.46	66.67	53.72% to 77.51%	41.67	19.33% to 68.05%	1.143
> 56.76	66.67	53.72% to 77.51%	58.33	31.95% to 80.67%	1.600
> 57.59	64.91	51.94% to 76.00%	58.33	31.95% to 80.67%	1.558
> 58.35	**64.91**	**51.94% to 76.00%**	**66.67**	**39.06% to 86.19%**	**1.947**
> 58.94	64.91	51.94% to 76.00%	75.00	46.77% to 91.11%	2.596
> 60.18	61.40	48.43% to 72.94%	75.00	46.77% to 91.11%	2.456
> 61.30	59.65	46.70% to 71.38%	75.00	46.77% to 91.11%	2.386
> 61.69	57.89	44.98% to 69.81%	75.00	46.77% to 91.11%	2.316
> 62.20	56.14	43.28% to 68.23%	75.00	46.77% to 91.11%	2.246
> 62.64	56.14	43.28% to 68.23%	83.33	55.20% to 97.04%	3.368
> 63.23	54.39	41.59% to 66.63%	83.33	55.20% to 97.04%	3.263
> 63.78	52.63	39.92% to 65.01%	83.33	55.20% to 97.04%	3.158
> 64.38	52.63	39.92% to 65.01%	91.67	64.61% to 99.57%	6.316
> 65.45	50.88	38.26% to 63.38%	91.67	64.61% to 99.57%	6.105
> 68.32	49.12	36.62% to 61.74%	91.67	64.61% to 99.57%	5.895
> 70.88	47.37	34.99% to 60.08%	91.67	64.61% to 99.57%	5.684
> 73.37	47.37	34.99% to 60.08%	100.0	75.75% to 100.0%	

> 76.17	45.61	33.37% to 58.41%	100.0	75.75% to 100.0%	
> 85.15	43.86	31.77% to 56.72%	100.0	75.75% to 100.0%	
> 96.66	42.11	30.19% to 55.02%	100.0	75.75% to 100.0%	
> 113.9	40.35	28.62% to 53.30%	100.0	75.75% to 100.0%	
> 145.3	38.60	27.06% to 51.57%	100.0	75.75% to 100.0%	
> 162.7	36.84	25.52% to 49.82%	100.0	75.75% to 100.0%	
> 169.0	35.09	24.00% to 48.06%	100.0	75.75% to 100.0%	
> 187.5	33.33	22.49% to 46.28%	100.0	75.75% to 100.0%	
> 224.8	31.58	21.00% to 44.48%	100.0	75.75% to 100.0%	
> 251.4	29.82	19.53% to 42.66%	100.0	75.75% to 100.0%	
> 263.0	28.07	18.08% to 40.83%	100.0	75.75% to 100.0%	
> 304.3	26.32	16.65% to 38.98%	100.0	75.75% to 100.0%	
> 336.6	24.56	15.23% to 37.10%	100.0	75.75% to 100.0%	
> 342.8	22.81	13.84% to 35.21%	100.0	75.75% to 100.0%	
> 368.6	21.05	12.47% to 33.29%	100.0	75.75% to 100.0%	
> 445.9	19.30	11.13% to 31.34%	100.0	75.75% to 100.0%	
> 531.8	17.54	9.819% to 29.37%	100.0	75.75% to 100.0%	
> 588.5	15.79	8.536% to 27.36%	100.0	75.75% to 100.0%	
> 640.8	14.04	7.287% to 25.32%	100.0	75.75% to 100.0%	
> 670.9	12.28	6.078% to 23.25%	100.0	75.75% to 100.0%	
> 686.8	10.53	4.914% to 21.12%	100.0	75.75% to 100.0%	
> 701.8	8.772	3.805% to 18.94%	100.0	75.75% to 100.0%	
> 728.4	7.018	2.763% to 16.70%	100.0	75.75% to 100.0%	
> 751.0	5.263	1.435% to 14.37%	100.0	75.75% to 100.0%	
> 754.5	3.509	0.6234% to 11.92%	100.0	75.75% to 100.0%	
> 761.9	1.754	0.08999% to 9.291%	100.0	75.75% to 100.0%	

Kaplan-Meyer Analysis of Overall Survival with value obtained from ROC analysis as cut-point

Log-rank (Mantel-Cox) test		
Chi square	2.966	
df	1	
P value	.085	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	2.682	
df	1	
P value	.102	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
IL-6low	Undefined	
IL-6high	16.50	
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.4757	2.102
95% CI of ratio	0.2043 to 1.108	0.9025 to 4.896
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.4327	2.311
95% CI of ratio	0.1873 to 0.9998	1.000 to 5.340

Kaplan-Meyer Analysis of Overall Survival with Median (MD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.2107	
df	1	
P value	.646	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.007225	
df	1	
P value	.932	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
sCTLA-4low	23.50	
sCTLA-4high	19.00	
Ratio (and its reciprocal)	1.237	0.8085
95% CI of ratio	0.5423 to 2.821	0.3545 to 1.844
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.8241	1.214
95% CI of ratio	0.3607 to 1.883	0.5311 to 2.773
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.8263	1.210
95% CI of ratio	0.3647 to 1.872	0.5341 to 2.742

Kaplan-Meyer Analysis of Overall Survival with Highest quartile (HQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.2253	
df	1	
P value	.635	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.4754	
df	1	
P value	.490	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
sCTLA-4low	19.00	
sCTLA-4high	28.00	
Ratio (and its reciprocal)	0.6786	1.474
95% CI of ratio	0.2519 to 1.828	0.5471 to 3.969
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	1.256	0.7964
95% CI of ratio	0.4904 to 3.215	0.3110 to 2.039

Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	1.268	0.7889
95% CI of ratio	0.4995 to 3.217	0.3109 to 2.002

Kaplan-Meyer Analysis of Disease Free Survival with value obtained from ROC analysis as cut-point

Log-rank (Mantel-Cox) test		
Chi square	2.248	
df	1	
P value	.134	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	2.257	
df	1	
P value	.133	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
sCTLA-4low	Undefined	
sCTLA-4high	12.00	
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.4720	2.119
95% CI of ratio	0.1769 to 1.260	0.7940 to 5.654
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.4402	2.272
95% CI of ratio	0.1675 to 1.157	0.8645 to 5.969

Kaplan-Meyer Analysis of Disease Free Survival with Median (MD) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.4124	
df	1	
P value	.521	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.001099	
df	1	
P value	.974	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
IL-6low	Undefined	
IL-6high	12.00	

Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.7282	1.373
95% CI of ratio	0.2766 to 1.917	0.5216 to 3.615
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.7337	1.363
95% CI of ratio	0.2835 to 1.899	0.5266 to 3.528

Kaplan-Meyer Analysis of Disease Free Survival with Highest quartile (HQ) as cut-point

Log-rank (Mantel-Cox) test		
Chi square	0.4124	
df	1	
P value	.521	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	0.001099	
df	1	
P value	.974	
P value summary	ns	
Are the survival curves sig different?	No	
Median survival		
TNF-a low	Undefined	
TNF-a high	12.00	
Hazard Ratio (Mantel-Haenszel)	A/B	B/A
Ratio (and its reciprocal)	0.7282	1.373
95% CI of ratio	0.2766 to 1.917	0.5216 to 3.615
Hazard Ratio (logrank)	A/B	B/A
Ratio (and its reciprocal)	0.7337	1.363
95% CI of ratio	0.2835 to 1.899	0.5266 to 3.528